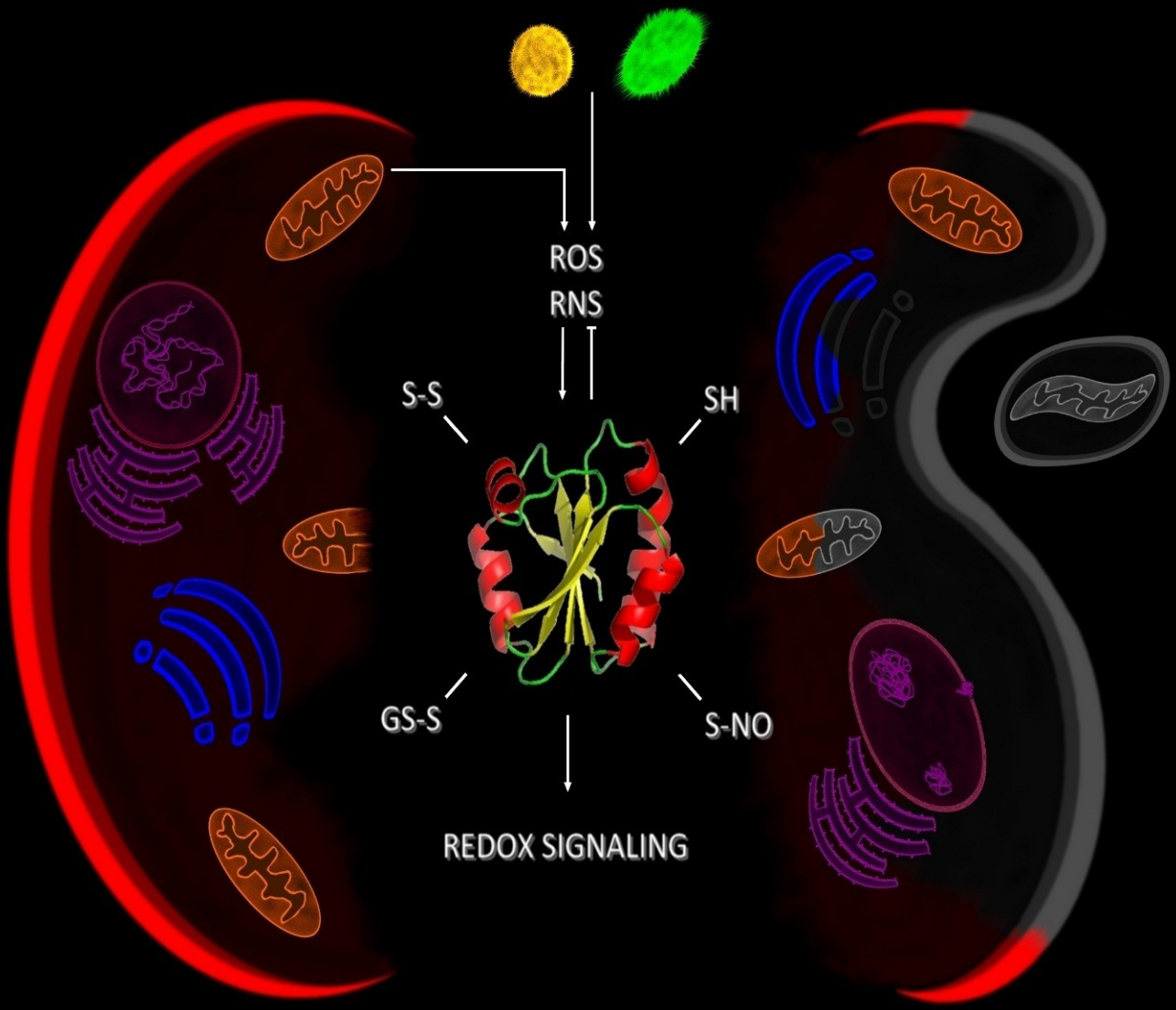
Institut für Klinische Zytobiologie und Zytopathologie, Prof. Dr. Roland Lill,

Medizinische Fakultät der Philipps-Universität Marburg

Thioredoxin family proteins in physiology and disease



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Department of Clinical Cytobiology and Cytopathology, Prof. Dr. Roland Lill,

Medical Faculty of the Philipps-Universität Marburg

Thioredoxin family proteins in physiology and disease

Eva-Maria Hanschmann

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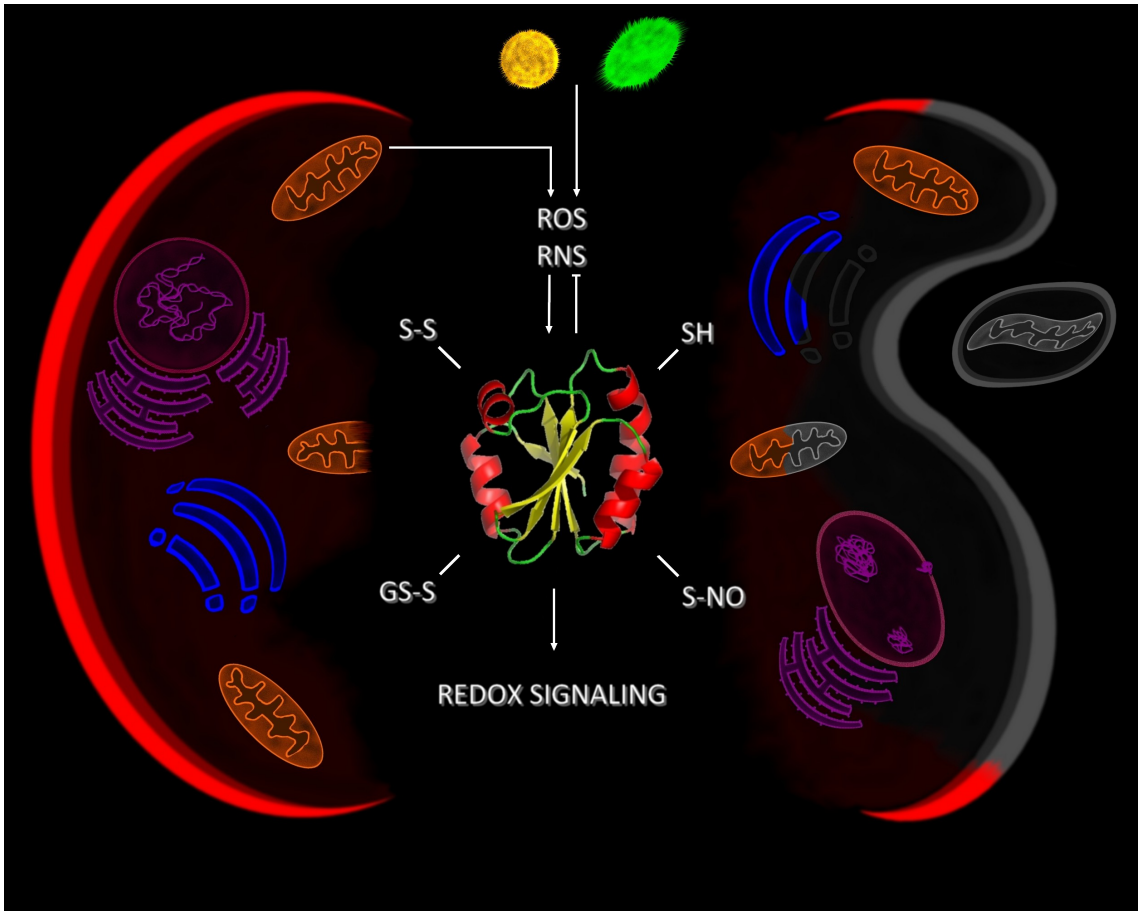

Numerous cellular processes are controlled by redox regulation via posttranslational modifications at thiol (SH) groups, i.e. for instance the formation of disulfide bonds (S-S), glutathionylation (S-SG) and nitrosylation (S-NO). Redox signaling is mediated by reactive oxygen species (ROS) and reactive nitrogen species (RNS) and members of the thioredoxin family of proteins. Exemplary human Trx1 is depicted in the cover picture. Redox signaling is essential for regulating the fate of a cell. Hence, disregulation of redox control has been implicated in various diseases leading e.g. to enhanced proliferation in cancer or to apoptosis in degenerative disorders.
The cover picture depicts a healthy cell to the left and a cell undergoing apoptosis to the right, characterised by condensation of DNA, membrane blebbing and phagocytosis of damaged organelles.

Front cover by Florian Knorz, according to the crayon drawing of Dr. Carsten Berndt, special issue on redox control of cell function, Biochimica et Biophysica Acta, (2008), 1780(11).

To my family,

especially to my grandfather Prof. Dr. Rolf Hanschmann

Summary

Proteins of the thioredoxin (Trx) family are ubiquitously expressed oxidoreductases. They use cysteinyl residues within their active site to modify substrate proteins posttranslationally by reduction/oxidation reactions or de-/glutathionylation. They play a crucial role in regulating cellular functions such as proliferation, differentiation and apoptosis. This thesis entitled "*Thioredoxin family proteins in physiology and disease"* focusses on these proteins, i.e. the Trx systems, the glutaredoxin (Grx) systems and the peroxiredoxins (Prxs). Because descriptions of cellular functions of redoxins are rare, we aimed at identifying new interaction partners and functions under physiological and hypoxic conditions in various cell culture and animal models.

We described the mitochondrial Grx2 as novel electron donor for Prx3, using a 2-Cys Prx-specific redox blot. Silencing the expression of Trx2, before this thesis the only known electron donor, or Grx2 in HeLa cells did not increase the level of oxidised Prx3, but simultaneous silencing did. Prx3 distribution in mouse tissues, was linked to the expression of either Trx2 or Grx2, depending on the cell type.

Knock-down of Trx1 and Grx2 affected iron regulation in HeLa cells. Especially Grx3 depletion resulted in strong defects in iron homeostasis, impairing cytosolic and mitochondrial heme- and iron-sulfur cluster containing proteins in HeLa cells and hemoglobin maturation in zebrafish.

Trx family proteins showed a tissue- and cell type-specific expression and distribution in the rat CNS and responded tissue- and cell type-specific to oxygen deprivation in numerous cell lines and models for perinatal asphyxia, renal ischemia/reperfusion (I/R) injury and transplantation of pancreatic β-cells.

Perinatal asphyxia in rat pups led to an immediate increase in the expression of Grx2, Trx1 and Trx2 four hours after induction of hypoxia. No significant longterm changes after 7 days were determined.

Renal I/R injury led to segment-specific alterations in the distribution and expression of Trx family proteins in the ischemic kidney, but also to systemic effects in the contralateral kidney. In proximal tubule cells, which can regenerate after an I/R insult, levels of Grx2, Prx3 and Prx6 were upregulated. Overexpression of these proteins in HEK293 cells had a positive impact on cell proliferation and survival under hypoxic conditions.

The protein levels of many cytosolic members of the Trx family were decreased, while the levels of mitochondrial proteins were increased in mouse models of pancreatic β-cell transplantation. Trx1 was secreted during hypoxia and reduced macrophage migration, potentially preventing the initiation of the immune response. Overexpression of Trx1 and Trx2 enhanced cell proliferation and survival following hypoxia/reoxygenation, by affecting the phosphorylation pattern of the MAP kinases ERK, JNK and p38.

This thesis emphasizes the concept of compartmentalised redox signaling and demonstrates not only the complexity of the Trx family proteins, but the species-, tissue- and cell type-specific responses to oxygen deprivation and the distinct contribution of the redoxins to controling the fate of a cell.

Zusammenfassung

Proteine der Thioredoxin (Trx) Familie sind ubiquitär exprimierte Oxidoreduktasen, die Cysteine innerhalb ihres aktiven Zentrums dazu nutzen, Proteine posttranslational durch Reduktions-Oxidations Reaktionen oder De-/Glutathionylierung zu modifizieren. Sie regulieren Prozesse wie Proliferation, Differenzierung und Apoptose. Diese Doktorarbeit mit dem Titel "*Thioredoxin family proteins in physiology and disease"* konzentriert sich auf die Trx Systeme, die Glutaredoxin (Grx) Systeme und auf die Peroxiredoxine (Prx). Da nur wenige zelluläre Funktionen dieser Proteine bekannt sind, galt es neue Interaktionspartner sowie Funktionen unter physiologischen und hypoxischen Bedingungen in verschiedenen Zell- und Tiermodellen aufzudecken.

Mittels eines 2-Cys Prx-spezifischen Redoxblots wurde das mitochondrielle Grx2 als Elektronendonor für Prx3 identifiziert. Die Verringerung der Proteinlevel von Trx2, dem vor dieser Studie einzigen bekannten Elektronendonor, oder Grx2 erhöhte die Menge an oxidiertem Prx3 nicht; jedoch die simultane Verringerung beider Redoxine. Die Verteilung von Prx3 in Geweben der Maus korrelierte, je nach Zelltyp, mit der Expression von Trx2 oder Grx2.

Die Reduktion der Trx1 und Grx2 Proteinmenge störte die Eisenregulation in HeLa Zellen. Die Verminderung des Grx3 Levels beeinträchtigte die Eisenhomöostase besonders, d.h. führte zu einer Reduktion der Aktivitäten von zytosolischen und mitochondriellen Häm- und Eisen-Schwefel Proteinen in HeLa Zellen, sowie zu einer verminderten Hämoglobin Produktion im Zebrafisch.

Proteine der Trx Familie zeigten eine gewebs- und zell-spezifische Expression und Verteilung im Zentralen Nervensystem der Ratte, sowie eine gewebs- und zell-spezifische Antwort auf Sauerstoffmangel in verschiedenen Zelllinien und Tiermodellen für perinatale Asphyxie, renale Ischämie/Reperfusion (I/R) und Betazell-Transplantation.

Perinatale Asphyxie in Rattenjungen führte zu einer direkten Erhöhung der Proteinlevel von Grx2, Trx1 und Trx2, vier Stunden nach Induktion der Hypoxie. Es konnten keine signifikanten Langzeitveränderungen nach sieben Tagen nachgewiesen werden.

Renale I/R führte zu segment-spezifischen Veränderungen in der Verteilung und Expression der Redoxine in der ischämischen und aufgrund systemischer Effekte in der kontralateralen Niere. In proximalen Tubuluszellen, die sich nach einem I/R Vorfall regenerieren können, wurden Grx2, Prx3 und Prx6 hochreguliert. Überexpression dieser

Redoxine in HEK293 Zellen führte zu einer erhöhten Proliferations- und Überlebensrate unter hypoxischen Bedingungen.
In Modellen für die Transplantation von β-Zellen des Pankreas wurden zytosolische Redoxine herunterreguliert und mitochondrielle Proteine hochreguliert. Trx1 wurde nach Hypoxie extrazellulär detektiert, wo es die Migration von Makrophagen verlangsamte und somit die Einleitung der Immunantwort zu unterdrücken scheint. Die Überexpression von Trx1 und Trx2 erhöhte die Zellproliferations- und Überlebensrate nach Hypoxie/Reoxygenierung, durch Veränderungen der Phosphorylierungen der MAP Kinasen ERK, JNK und p38.

Diese Arbeit unterstreicht das Konzept des kompartimentalisierten Redox-Signalings und zeigt nicht nur die Komplexität der Trx Familie, sondern spezies-, gewebs- und zell-spezifische Antworten auf Hypoxie und den unterschiedlichen Beitrag der Redoxine an der Kontrolle über das Zellschicksal.

Abbreviations

ALP	Alkaline phosphatase
Ask1	Apoptosis-signal regulating kinase 1
Caspase	Cysteine-dependent aspartate-directed protease
Cys	Cysteine
DTNB	5,5'-Dithio-bis-(2-Nitrobenzoic acid)
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellularly regulated kinase
FCS	Fetal calf serum
FeS	Iron-sulfur
GPAT	Glutamine phosphoribosylpyrophosphate amidotransferase
GPx	Glutathione peroxidase
GR	Glutathione Reductase
Grx	Glutaredoxin
GSH	Glutathione
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HIF	Hypoxia-induced factor
Hsp	Heat shock protein
IHC	Immunohistochemistry
I/R	Ischemia/Reperfusion
IRE	Iron responsive element
IRP	Iron regulatory protein
JNK	c-Jun N-terminal kinase

LPS	Lipopolysaccharide
MAP	Mitogen-activated protein
mTAL	Medullary thick ascending limb
MTS	Mitochondrial translocation signal
NADPH	Nicotine adenine dinucleotide phosphate
NEM	N-ethylmalemeide
NFκB	Nuclear factor-κB
NO	Nitric oxide
PAGE	Polyacrylamidgelelectrophoresis
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PFA	Paraformaldehyde
PICOT	Protein interacting cousin of thioredoxin
Prx	Peroxiredoxin
RNR	Ribonucleotide reductase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
siRNA	Small interfering RNA
TCEP	Tris(2-carboxyethyl)phosphine
TfR	Transferrin receptor
Tnfα	Tumor necrosis factor alpha
Trx	Thioredoxin
TrxR	Thioredoxin reductase
Txnip	Thioredoxin interacting protein
γGCS	Glutamate cysteine synthetase

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1. Introduction

In all kingdoms of life cellular processes are redox controlled. Lipids, nucleic acids and proteins can be redox modified, but only the latter are regulated by reversible modifications at thiol groups (Schafer and Buettner, 2001). The thioredoxin (Trx) family proteins constitute key players in maintaining cellular redox homeostasis and redox signaling (Nordberg and Arnér, 2001), (Lillig et al. 2008). Cell organelles are equipped with distinct sets of these proteins, or reductants in general, and exhibit several sources of oxidants, leading to compartmentalised redox signaling (Jones, 2010) and a broad, but specific range of substrates and functions for redox regulation. Disregulation of redox signaling and an increased formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are correlated with numerous pathologies, including cancer, cardiovascular- and degenerative disorders (Lillig and Holmgren, 2007).

1.1 Redox regulation of proteins

Protein activity, structure, subcellular distribution and interactions with substrates are controlled by posttranslational modifications. These modifications include phosphorylation, acetylation, glycosylation, methylation and ubiquitylation (reviewed in Walsh et al. 2005). In addition, oxidative modifications display alterations at redox reactive amino acids. Here, cysteine (Cys) constitutes the main target, but also methionine and selenocysteine can undergo redox reactions. Cys is a polar amino acid, commonly found on the surface of proteins, containing a reactive thiol group. Adjacent to basic amino acids, thiol groups are extremely reactive at physiological pH, due to a lowering of the pKa from typically eight to between five and seven (Foster et al. 2009). The human genome encodes for approximately 24000 proteins, comprising about 214000 cysteine residues (Jones, 2010). Miseta and Csutora stated that the Cys content and the general number of proteins containing at least one Cys increased along with animal evolution, indicating a development of signaling and regulatory functions of this amino acid (Miseta and Csutora, 2000), (Fomenko et al. 2008). Cys is particularly important for stabilizing the structure of proteins or crosslinking different proteins via the oxidation of two Cys residues to a cystine or a so called disulfide bridge. Single Cys residues can be modified by glutathionylation, the formation of a disulfide bond with a

glutathione (GSH) molecule. They can also be nitrosylated by nitric oxide (NO), sulfhydrated by H_2S, can form thiolhemiacetals in the presence of aldehydes or can be alcylated by acetyl-CoA. Another feature of the amino acid is the ability to coordinate metal clusters, such as iron-sulfur (FeS) clusters (Jones, 2008). Sustained oxidative conditions can lead to irreversible modifications i.e. the overoxidation to sulfinic or sulfonic acid, changing the activity and/or function of a protein unalterably. Both, reversible and irreversible modifications of Cys residues are summarized in Figure 1.

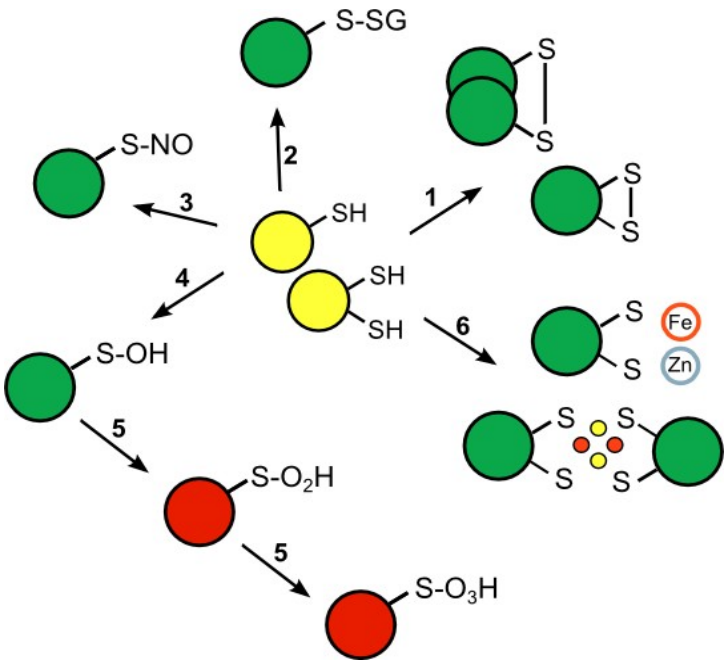

Figure 1: Cysteine can be posttranslationally modified. The reactive thiol group is posttranslationally modified reversibly (green) and irreversibly (red): (1) formation of disulfide bridges, (2) glutathionylation, (3) nitrosylation, (4) formation of sulfenic acid and latter (5) sulfinic and sulfonic acid, (6) coordination of metals/ metal clusters. These modifications regulate proteins in terms of structure, intracellular distribution, protein interaction and activity and thereby affect a proteins function(s).

1.1.1 Redox regulated cellular pathways

Basic requirements for signaling cascades are substrate specificity and rapid, reversible changes in protein activity following specific stimuli, leading to a distinct outcome in cellular function (Janssen-Heininger et al. 2008). Redox signaling fulfils these criteria. Disulfide oxidoreductases of the Trx family and ROS, such as hydrogen peroxide (H_2O_2), mediate redox regulation of proteins. Cells are surrounded by a plasma membrane, which separates the intracellular reducing space from the extracellular oxidising environment. In this lipid bilayer different kinds of polypeptides are embedded including adhesion proteins, ion channels and membrane receptors, triggering intracellular signaling cascades in response to environmental factors. Membrane proteins are generally known to contain disulfides, which are stable in the extracellular room. However, several reports show that redox regulation of protein

function is not restricted to the intracellular space (Moriarty-Craige and Jones, 2004). Glycerophosphodiester phosphodiesterases (GDE) constitute a family of transmembrane signaling proteins, which regulate differentiation processes in diverse cellular contexts. Yan and collegues have shown that the peroxidase peroxiredoxin (Prx) 1 reduces an intermolecular disulfide of GDE2, thereby controling the differentiation of spinal motor neurons (Yan et al. 2009).

A similar regulatory mechanism was found in the family of multimeric transient receptor potential (TRP) channels, which transports cations including Ca^{2+}, Na^{+} and Mg^{2+} ions across the membrane (Venkatachalam and Montell, 2007). Several of these channels were shown to possess two conserved Cys residues. Xu *et al.* treated HEK293 cells, expressing TRPC5-TRPC1 channels with the reductants dithiothreitol (DTT) and Tris(2-carboxyethyl)phosphine (TCEP), as well as extracellular with reduced Trx1, demonstrating a reduction of an intramolecular disulfide between the conserved thiol groups and a subsequent activation of the ion channel (Xu et al. 2008). Hara *et al.* showed that the addition of oxidants such as H_2O_2 activated the LTRPC2 channel in HEK293 cells, introducing the channel as a mediator of cell death due to a cellular cation-overload in response to a disregulated redox state (Hara et al. 2002). Other targets of hydrogen peroxide include molecular chaperones, peptidases and tyrosine phosphatases. Heat shock proteins (Hsp) are cellular chaperones facilitating protein folding. Bacterial Hsp33 is a redox sensitive chaperone, which refolds proteins coping with severe oxidising conditions, when reduced to its monomeric form. Monomeric Hsp33 contains four Cys residues coordinating a zinc atom. Upon oxidising conditions, the zinc is lost and intramolecular disulfides are formed, initiating chaperone activity (Jakob et al. 1999). The induction of an immediate heat shock response is essential for cell survival. Besides disulfide formation, several chaperones have been shown to be regulated by reversible glutathionylation, reviewed in Berndt et al. 2008 or nitrosylation (Martínez-Ruiz et al. 2005). The oxidoreductase glutaredoxin (Grx) 1 was shown to reduce glutathionylated human Hsp70, attenuating protein activity (Hoppe et al. 2004).

Cys-dependent aspartate-directed proteases (Caspases) catalyse the degradation of proteins by hydrolysis of peptide bonds at specific amino acids. They possess a Cys residue within their conserved Gln-Ala-Cys-X-Gly active site motif. Reduction or rather de-nitrosylation leads to activation of the protease, following a pro-apoptotic stimulus. It was shown that H_2O_2 exhibits caspase activation at low concentrations, but

induces necrosis at higher levels (Saito et al. 2006). Furthermore, there are studies showing regulatory mechanisms of Trx family proteins. Mitchell *et al.* revealed an anti-apoptotic effect of Trx1 in Jurkat cells, due to S-nitrosylation of pro-caspase-3 (Mitchell et al. 2007). Pan and Berk demonstrated that S-glutathionylation renders caspases inactive. Grx1 catalyses the de-glutathionylation of caspase-3 functioning in tumor necrosis factor α (TNFα)-induced apoptosis (Pan and Berk, 2007).

Tyrosine phosphatases, enzymes which catalyse the de-phosphorylation of proteins on tyrosine residues, are regulated in a similar way. Oxidation of a redox active Cys in their active site renders them inactive, leading to an increase in phosphorylated target proteins. It is well documented that growth factors such as the epidermal growth factor lead to intracellular production of H_2O_2 followed by the oxidation of the cysteinyl residue (Lee et al. 1998), (Xu et al. 2002). The antagonists of phosphatases are kinases, which catalyse the phosphorylation of target proteins. Mitogen-activated protein (MAP) kinases, including apoptosis signal regulating kinase 1 (Ask1), extracellularly regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (reviewed in Matsuzawa and Ichijo, 2008) are key players in signaling cascades regulating cell proliferation, differentiation and apoptosis. These proteins are not only regulated by de-phosphorylation, but also by redox modifications at conserved Cys residues. Ask1 is a known substrate of Trx1. Strictly speaking it is not redox regulated, but it is kept in an inactive state by reduced Trx1. Upon oxidising conditions, Trx1 becomes oxidised, releasing Ask1, which in turn induces apoptosis (Saitoh et al. 1998). Ivarsson *et al.* studied redox regulation of exocytosis in rat pancreatic β-cells. Treatment with glucose leads to secretion of insulin and at the same time increases the NADPH/ $NADP^+$ ratio. Direct addition of NADPH leads to exocytosis of insulin granules itself. Mediators of this effect seem to be the NADPH-dependent oxidoreductases Grx and Trx (Ivarsson et al. 2005). Many transcription factors have been described to be redox regulated via Cys residues at their DNA-binding domain, for instance nuclear factor-κB (NFκB) and activator protein-1 (AP-1), which are involved in the response to various stimuli including alterations of the redox state (Sen and Packer, 1996). NFκB, which is important for cell adhesion, growth control, immune modulation and inflammatory response (reviewed in Luqman and Pezzuto, 2010) has a critical Cys which can be reduced by nuclear Trx1, inducing transcription (Hayashi et al. 1993). Cytosolic Trx1 seems to have the opposite effect, by promoting the binding of the inhibitor protein IκB to NFκB (Hirota et al. 1999).

The gene encoding heme oxygenase-1 (HO-1) e.g. contains NFκB regulatory elements in the promotor region (Naidu et al. 2008). HO-1 is a protein catalysing the degradation of heme. Iron is essential for the cell and is needed for the synthesis of heme, FeS proteins and as cofactor. However, iron homeostasis has to be tightly controlled to avoid iron deficiency or iron overload. Cellular iron levels are controlled by the two iron regulatory proteins IRP1 and IRP2, which bind to cis-regulatory iron responsive elements (IRE) on the mRNAs of the iron import transferrin receptor (TfR), the iron storage protein ferritin and the iron export protein ferroportin (Muckenthaler et al. 2008), (Wallander et al. 2006). IRP1 is an iron-sulfur protein. Upon iron deficiency it loses the cluster, binds to IREs where it for instance stabilizes the mRNA of the TfR and represses the translation of ferritin. Similarly, the cytosolic monothiol Grx3 and Grx4 in yeast form a FeS-bridged complex, which was also shown to act as iron sensor. Depletion of Grx3 and Grx4 in yeast cells, leads to impaired intra-compartimental trafficking of iron and the disturbed synthesis of heme- and FeS cluster-containing proteins

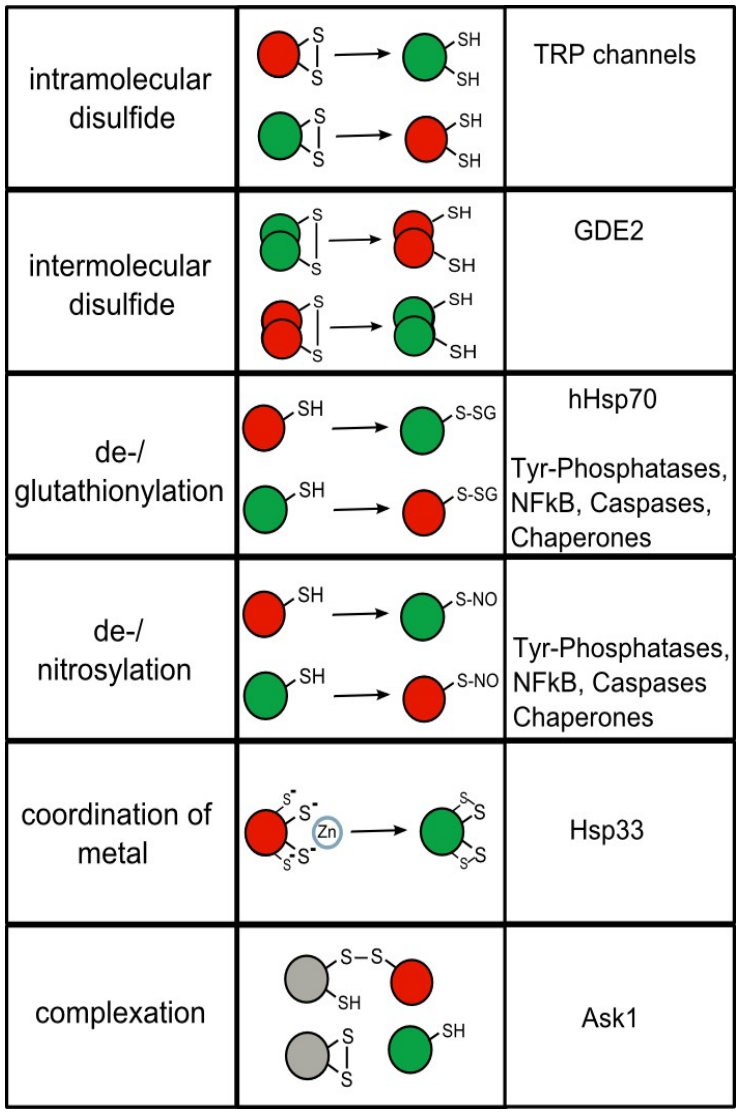

Figure 2: Reversible posttranslational modifications regulate protein function. Caspases, channel proteins, chaperones and phosphatases can be regulated via intra- and intermolecular disulfides, de-/glutathionylation and de-/nitrosylation. Hsp33 coordinates a zinc atom in its inactive form, which is lost for protein activation. The kinase Ask1 is kept in an inactive complex with Trx1. Upon oxidation it gets released and becomes activated (red = inactive, green = active).

(Mühlenhoff et al. 2010). Yeast mutants lacking the mitochondrial monothiol Grx5 are characterized by the accumulation of iron and inactivated FeS cluster containing proteins (Kim et al. 2010).

1.2 Thioredoxin family proteins in mammals

The Trx family comprises Trx - the founding member of this protein family - glutaredoxins, peroxiredoxins, protein disulfide isomerases (PDI), glutathione-peroxidases (GPx), glutathione-S-transferases (GST) and chloride channels. All these proteins share a common structural motif, the Trx fold, consisting of a central core of four-stranded β-sheets surrounded by three α-helices, and the conserved active site Cys-X-X-Cys (Martin, 1995). In the following, the mammalian proteins analysed in this study, i.e. Trxs, Grxs and Prxs and related proteins, will be introduced and discussed in terms of structure, intracellular localisation (summarized in Figure 7), substrates and functions.

1.2.1 Thioredoxin system

The mammalian Trx system consists of Trx, the selenoprotein thioredoxin reductase (TrxR) and NADPH (Figure 3). It exerts a variety of functions due to a broad substrate specificity of Trx and TrxR itself. The Trx system is essential for DNA synthesis (Laurent et al. 1964), proliferation, protection against apoptosis (Powis et al. 1998) and regulation of transcription (Abate et al. 1990), (Matthews et al. 1992). It is an essential antioxidative system, regulating the redox state of proteins and modulating signaling cascades and the immune response, by reducing substrates via the dithiol mechanism (Holmgren, 1985). The N-terminal active site Cys of Trx is characterized by a low pKa value, which allows it to initiate a nucleophilic attack at a target dithiol. A covalently bound mixed dithiol is

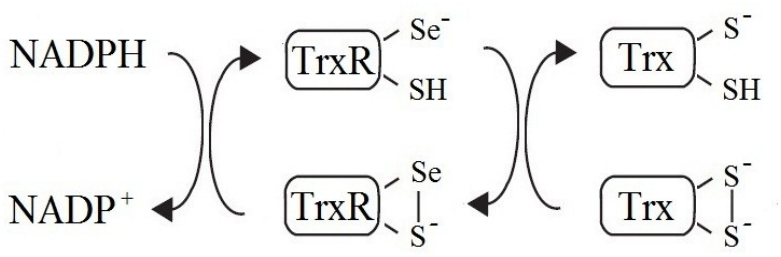

Figure 3: Electron flow of the thioredoxin system. NADPH reduces the selenoprotein TrxR, which in turn reduces Trx.

generated, which is reduced by the C-terminal Cys yielding a reduced substrate and an oxidised Trx. The protein disulfide in the active site of Trx is reduced by TrxR, receiving electrons from NADPH (Holmgren, 1995). In mammals two Trx systems exist, a cytosolic (Trx1, TrxR1) and a mitochondrial system (Trx2, TrxR2). Reduced Trx is inhibited by Txnip (thioredoxin interacting protein), which structurally is not part of the Trx family (Nishiyama et al. 1999).

1.2.1.1 Thioredoxin reductase

Thioredoxin reductase is a NADPH-dependent flavoenzyme and is one of 25 selenoproteins in humans (Kryukov et al. 2003). The 55-60 kDa protein is a homo-dimer in a head-to-tail conformation, with every subunit consisting of a flavin adenine dinucleotide (FAD) domain, a NADPH binding domain and an interface domain. TrxR contains two active sites. The active site motif Gly-Cys-Sec-Gly is found at the N-terminus; the active site motif Cys-Val-Asn-Val-Gly-Cys at the C-terminus, in close proximity to the FAD domain. TrxR is closely related to glutathione reductase (GR); both belong to a family of homo-dimeric pyridine nucleotide-disulfide oxidoreductases (Arnér, 2009). Even though these enzymes possess similar structures, TrxR contains the selenocysteine in a 16 amino acid extension, which is not present in GR (Urig et al. 2006), (Zhong et al. 1998). Due to high accessibility and reactivity of selenocysteine, TrxR has a broad substrate specificity, with Trx being the main target. Other substrates include PDI (Lundström and Holmgren, 1990), glutaredoxin 2 (Johansson et al. 2004) and dehydroascorbate (May et al. 1997). In mammals, there are three thioredoxin reductases, the cytosolic TrxR1, the mitochondrial TrxR2 and the testis-specific TrxR3, which is mainly expressed in germ cells and will not be further discussed within this thesis. Different splice variants of TrxR have been described, giving rise to different proteins with distinct functions (Rundlöf et al. 2004), (Turanov et al. 2006). Knock-out of either TrxR1 or TrxR2 in mice causes embryonic lethality. TrxR1 expression is evidently essential during embryogenesis in most tissues except for the heart (Jakupoglu et al. 2005), while TrxR2 is crucial in both hematopoiesis and heart function (Conrad et al. 2004).

1.2.1.2 Thioredoxin

The 12 kDa Trx was discovered 1964 in *E. coli* as electron donor for ribonucleotide reductase (RNR), the enzyme essential for DNA synthesis in all organisms (Laurent et

al. 1964), (Moore et al. 1964), and was recognized as general cellular disulfide reductase in 1979 (Holmgren, 1979). The oxidoreductase contains the active site motif Cys-Gly-Pro-Cys (Holmgren, 1968), which is highly conserved throughout different species from bacteria to human (Eklund et al. 1991). It is a primarily cytosolic protein, which can translocate into the nucleus upon various stimuli (Hirota et al. 1999) or can be secreted (Rubartelli et al. 1992). Mitochondrial Trx2 possesses the active site motif of Trx1, but lacks additional structural cysteines. Both proteins share 35 % sequence homology and similar catalytic properties *in vitro* (Spyrou et al. 1997). Knock-out of either Trx1 or Trx2 in mice is lethal. Trx1 expression seems to be important for early differentiation and morphogenesis of the embryo (Matsui et al. 1996), while Trx2 knock-out mice show an increased apoptosis rate coinciding with the maturation of mitochondria around day 12.5 (Nonn et al. 2003). As described above, Trx1, as well as Trx2, regulate Ask1 and thereby the p38 and the JNK pathway (Saitoh et al. 1998), (Saxena et al. 2010). Trx1 regulates the activity of various transcription factors which have Cys residues in their DNA binding domains, including for instance NFκB. Another group of substrates are the related Prxs, that constitute the major cellular peroxidases (Rhee et al. 1999), (Berggren et al. 2001).

1.2.1.3 Txnip

Txnip, also known as TBP2 (thioredoxin binding protein 2) (Nishiyama et al. 1999) or VDUP1 (Vitamin D upregulating protein 1) (Schulze et al. 2002), was discovered in a yeast two-hybrid system as binding partner of Trx (Nishiyama et al. 1999), (Yamanaka et al. 2000). Structurally, Txnip is assigned to the arrestin superfamily of regulatory proteins, which is characterized by two wing-like arrestin domains forming a central core that binds to phosphates on activated receptors. The interaction between the 50 kDa Txnip and Trx depends on a disulfide linkage between reduced active site cysteines of Trx (Nishiyama et al. 1999) and two Cys residues of the inhibitor protein (Patwari et al. 2006). These residues are not conserved in the arrestin family, giving Txnip a prominent and unique role. Txnip regulates the Trx1/Ask1-dependent apoptosis pathway (Chen et al. 2008). This interaction was recently also shown for mitochondrial Trx2 in pancreatic β-cells, which is regulated by Txnip shuttling between cytosol, nucleus and mitochondria (Saxena et al. 2010). The pro-apoptotic role was analysed by Minn *et al.,* showing that the overexpression of Txnip induces apoptosis through an increase in the Bax/Bcl-2 ratio, and caspase-3 expression (Minn et al. 2005). By binding

Trx and inhibiting its disulfide reductase activity, Txnip is involved in various cellular processes including cell proliferation and apoptosis. However, Patwari and coworkers demonstrated that the function of Txnip is not necessarily connected to the binding of Trx. The overexpression of wildtype Txnip and a cysteine-serine mutant protein, which is not able to bind Trx, inhibited glucose uptake in mature adipocytes in equal measure (Patwari et al. 2009). Txnip has been shown to be dramatically induced by glucose, suppressed by insulin and strongly upregulated in diabetes (Parikh et al. 2007). This function as regulator of glucose metabolism is underlined by the fatal phenotype of Txnip knock-out mice, which have fasting hypoglycaemia with increased glucose uptake in peripheral tissues (Chutkow et al. 2008), (Hui et al. 2004). In addition, these mice have an impaired lipid metabolism (Oka et al. 2006).

1.2.1.4 Nucleoredoxin

Nucleoredoxin (Nrx) contains two N-terminal Trx-like domains with the active site motif Trp-Cys-Pro-Pro-Cys and a C-terminal PDI-like Trx domain without any redox active Cys residues (Funato and Miki, 2007). It is located in the cytosol and in the nucleus, even though no nuclear localisation sequence was found. Nrx was shown to bind to the dishevelled (Dvl) protein, suppressing the Wnt/β-catenin pathway, which is essential for embryonic development. The active site motif of Nrx was shown to be essential for Nrx-Dvl interaction (Funato et al. 2006). A similar regulatory function was described for Toll-like receptor 4 signaling (Hayashi et al. 2010). Nrx knock-out mice are characterised by skeletal and cardiovascular defects. Funato *et al.* showed that Wnt signaling is increased in mouse $Nrx^{-/-}$ osteoblasts, confirming previous findings in cell culture and *Xenopus laevis*. However, they demonstrated decreased activity in $Nrx^{-/-}$ cardiocytes. In this study Nrx was introduced as a novel stabilizing protein of Dvl, protecting it from ubiquitination and thereby retaining a pool of Dvl, which is responsive to Wnt signaling (Funato et al. 2010).

1.2.2 Peroxiredoxins

Cells contain three types of peroxidases, i.e. Prxs, catalases and glutathione peroxidases. Prxs are unique among these enzymes because they catalyse the reduction of peroxides using their cysteine(s)-containing active site. Prxs are highly abundant proteins, which can account for up to 1 % of soluble cellular proteins (Chae et al. 1999), (Wood et al. 2003). Mammalian cells contain six Prxs, which are divided into three

groups, based on their structure and catalytic mechanism: 2-Cys Prxs (Prx1-4), atypical 2-Cys Prxs (Prx5), 1-Cys Prx (Prx6) (Seo et al. 2000), (Rhee et al. 2001). 2-Cys Prxs contain conserved C- and N-terminal Cys residues and exist as homo-dimers and high oligomers, whereas atypical 2-Cys and 1-Cys Prxs only contain the N-terminal Cys and do not form dimers. During the reduction of peroxides, the N-terminal Cys of 2-Cys Prxs is oxidised to sulfenic acid which forms an intermolecular disulfide with the C-terminal "resolving" Cys of another Prx subunit and an intra-molecular disulfide with a non conserved Cys at the C-terminus in the case of atypical 2-Cys Prxs. 2-Cys and atypical 2-Cys Prxs are substrates for the Trx system (Figure 4).

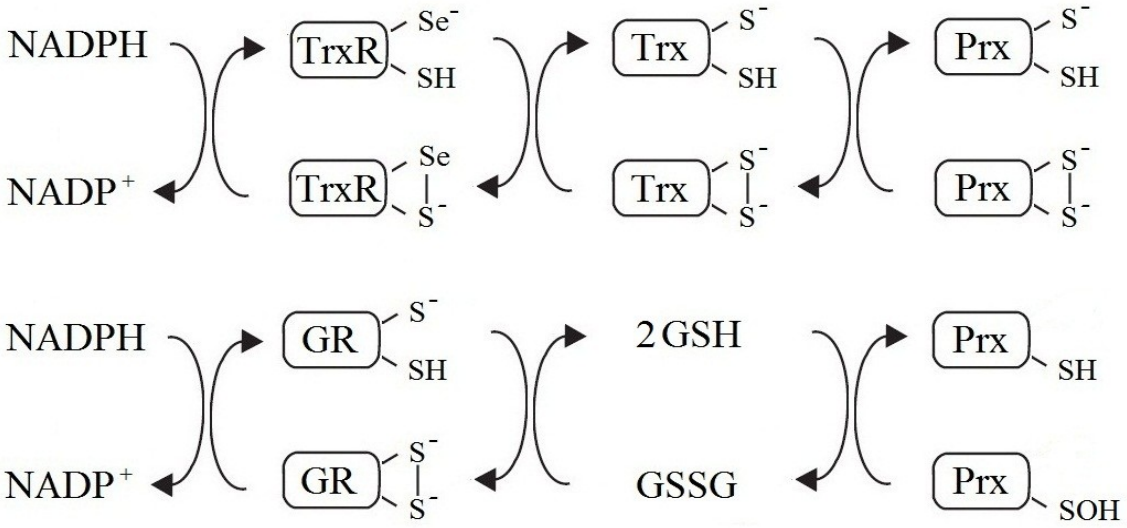

Figure 4: Electron donors for peroxiredoxins. 2-Cys Prxs are reduced by the Trx system (upper panel), whereas 1-Cys Prxs are reduced by GSH (lower panel).

1-Cys Prxs lack additional cysteines and are reduced by GSH (Rhee et al. 2001). Over-oxidised Prxs are reduced by sulfiredoxins (Woo et al. 2005). Prxs are 20-30 kDa proteins, varying in the subcellular localization (Hofmann et al. 2002), (Wood et al. 2003). In addition to the function as peroxidase, alternative functions have been proposed, including the role as molecular chaperones (Kumsta and Jakob, 2009), (Jang et al. 2004) and controling the levels of the cellular signaling molecule H_2O_2. Prx1 is mainly localized in the cytosol and in the nucleus. It was also found to be secreted (Chang et al. 2005). Prx1 knock-out mice are viable, but develop hemolytic anemia with oxidative damaged erythrocytes and bone marrow cells after nine months and develop significantly more malignant tumors with age (Neumann et al. 2003). Prx2 is present in the cytosol and the nucleus. Prx2-deficient macrophages show an enhanced inflammatory response when stimulated with lipopolysaccharides (LPS) (Yang et al.

2007). Knock-out mice are viable but develop a splenomegaly caused by congestion of the red pulp with hemosiderin accumulation and show morphologically different erythrocytes (Lee et al. 2003). Functions of Prx2 in inflammation and the life cycle of erythrocytes have been proposed. Knock-out mice of the mitochondrial Prx3 develop normally but show a reduction in body weight. These mice are believed to have higher levels of intracellular ROS and were shown to be more susceptible to LPS-induced lung damage (Li et al. 2007). Prx4 is found in the cytosol and in the endoplasmic reticulum. It contains a leader-peptide, which is believed to be essential for protein secretion (Okado-Matsumoto et al. 2000). Mice lacking Prx4 were viable and fertile, but showed a size-reduction of male testicles due to an elevated apoptosis rate of spermatogenic cells (Iuchi et al. 2009). Prx5 was found in cytosol, mitochondria and peroxisomes. Overexpression of Prx5 prevents peroxide-induced mitochondrial DNA damage (Zhou et al. 2000). Cytosolic Prx6 is not essential in mice, but macrophages without detectable levels of Prx6 were characterised by higher cellular levels of H_2O_2 and an elevated apoptosis rate (Wang et al. 2006).

1.2.3 Glutaredoxin system

Grxs and Trxs share a lot of structural and functional features. However, Grxs are more versatile. They do not only catalyse the reduction of disulfides, but also the protein de-/glutathionylation. Grxs display unique features in their Trx-folded structures: a GSH binding site (Bushweller et al. 1994) and a hydrophobic surface area for substrate interaction (Xia et al. 1992). As depicted in Figure 5, GSH constitutes the main electron

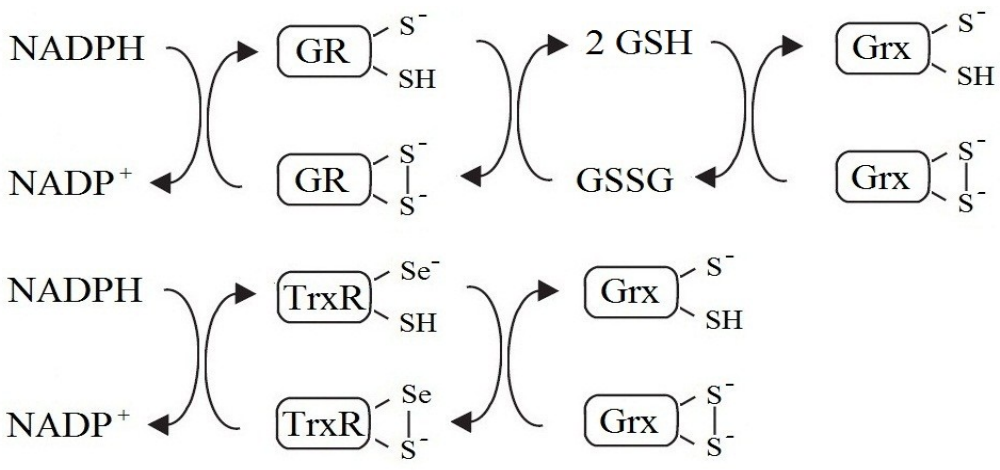

Figure 5: Electron flow of the glutaredoxin system. Grx is reduced by GSH, which is reduced by GR receiving electrons from NADPH. In the case of Grx2, TrxR can act as alternative electron donor.

donor for the oxidoreductase Grx, which in turn is reduced by glutathione reductase and NADPH (Meister and Tate, 1976). In addition, Grx2 can receive electrons from TrxR (Johansson et al. 2004).

Grxs contain the active site motif Cys-X-X-Cys, using both thiol groups for the catalysis of disulfides via the dithiol mechanism and only the N-terminal active site thiol for the reduction of glutathione-mixed disulfides and the catalysis of de-/glutathionylation of proteins via the monothiol mechanism (Lillig et al. 2008), (Yoshitake et al. 1994), (Yang and Wells, 1991). Besides the so called dithiol Grxs, Grxs with the active site motif Cys-X-X-X were classified as monothiol Grxs, which so far have not been shown to be catalytically active, but function in iron homeostasis and in the biosynthesis of FeS proteins (Herrero and de la Torre-Ruiz, 2007). So far, four Grxs have been discovered in mammals: Grx1, Grx2, Grx3 (also known as protein interacting cousin of Trx – PICOT) and Grx5.

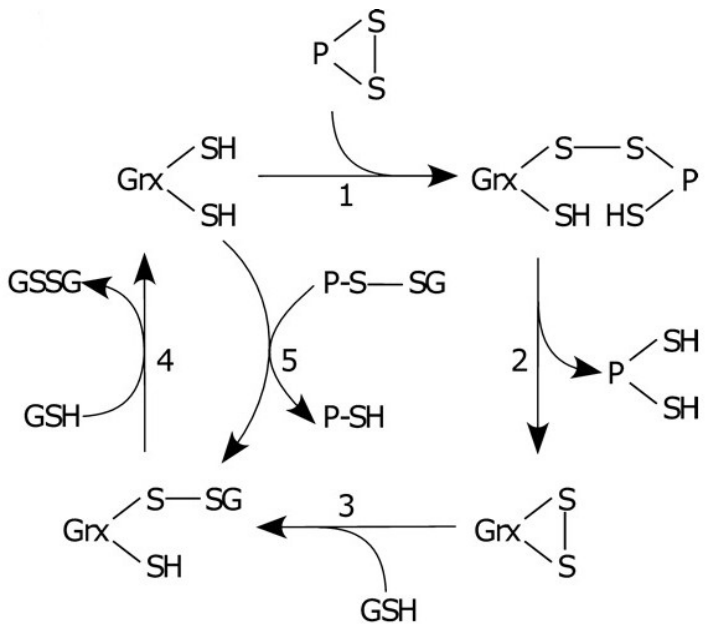

Figure 6: Reaction mechanisms. The dithiol mechanism, depends on both active site cysteines. The N-terminal Cys forms a protein-mixed disulfide (1), which is reduced by the C-terminal Cys (2). Grx is reduced by GSH (3,4). The monothiol mechanism depends on the N-terminal Cys, forming a Grx-GSH-mixed disulfide (5), which is reduced by GSH (4) (Berndt *et al.* 2007).

1.2.3.1 Glutathione

GSH is a tripeptide which is synthesized in the cytosol in two ATP-dependent reactions. Glutamate and cysteine are linked in the rate-limiting step by the enzyme glutamate cysteine synthetase (γGCS), which consists of a catalytic and a modulatory subunit. Mice lacking the catalytic subunit are embryonically lethal (Dalton et al. 2000). Glycine is attached to γ-glutamyl cysteine by the γ-glutamyltranspeptidase. 85-90 % of the synthesized GSH build the cytosolic GSH pool, the other 10-15 % are transported into distinct organelles, including mitochondria, where they build different, separated redox

environments (Griffith and Meister, 1985). GSH exists in a reduced and an oxidised state (GSSG), with GSSG being reduced by GR. It constitutes the major cellular thiol compound with intracellular concentrations in the millimolar range. GSH and GSSG act as buffer for the intracellular redox state of proteins. GSH scavenges free radicals either directly or as cofactor for GSTs, GPxs or as electron donor for the reduction of Grx, ascorbic acid and vitamin E (reviewed in Forman et al. 2009). It is used to detoxify xenobiotics and protects important thiols in proteins from oxidation by glutathionylation (Fratelli et al. 2004), which above that regulates protein function in processes including proliferation, differentiation and metabolism (Lind et al. 2002).

1.2.3.2 Glutathione reductase

Glutathione reductase is a 102 kDa enzyme, belonging to the pyridine nucleotide disulfide oxidoreductase family. Like TrxR, GR is a homo-dimer in a head-to-tail conformation, with every subunit consisting of a FAD domain, a NADPH binding domain and an interface domain (Karplus and Schulz, 1987). The flavoenzyme catalyses the reduction of GSSG to GSH using the N-terminal active site motif Cys-Val-Asn-Val-Gly-Cys and is highly conserved in all kingdoms of life. However, there are species that do not depend on the enzyme for maintaining the GSH redox state. For instance, in *Drosophila melanogaster* Trx reduces GSH (Kanzok et al. 2001). NADPH donates two electrons to the FAD domain, which are transferred to the active site at the FAD binding domain. These electrons are transferred to GSSG reducing it to two molecules of GSH. GR is located in the cytosol and mitochondria (Taniguchi et al. 1986), reducing different pools of GSH.

1.2.3.3 Glutaredoxin

The dithiol 12 kDa Grx1 is mainly localized in the cytosol, but can be translocated into the nucleus or exported from the cell (Björnstedt et al. 1994). Grx1 has the active site motif Cys-Pro-Tyr-Cys. The oxidoreductase regulates the activity of various proteins, including RNR (Holmgren, 1976), Ask1 (Murata et al. 2003) and NFκB (Daily et al. 2001). Mice lacking Grx1 are vital, although no compensatory de-glutathionylation activity could be detected (Ho et al. 2007). The dithiol Grx2 is located in mitochondria, but different cancer/testis-specific isoforms, restricted to the cytosol have been described in mouse and human (Hudemann et al. 2009), (Lönn et al. 2008). Grx2 has a molecular mass of 14 kDa and shares 34 % sequence homology with Grx1. The single

amino acid change within the active site motif Cys-Ser-Tyr-Cys enables the protein to receive electrons from TrxR (Johansson et al. 2004) and is essential for coordination of a [2Fe2S] cluster (Berndt et al. 2007), (Lillig et al. 2005). Grx2 is involved in mitochondrial redox regulation and antioxidant defence, reducing disulfides and GSH-mixed disulfides. It was furthermore shown to catalyse reversible oxidation and glutathionylation of membrane proteins (Beer et al. 2004). Grx2 stimulates proliferation and protects cells against apoptosis (Enoksson et al. 2005), (Lillig et al. 2004). The monothiol Grx3 is a multi-domain protein, containing one C-terminal Trx domain with the active site Ala-Pro-Gln-Cys and two N-terminal monothiol domains with the active site Cys-Gly-Phe-Ser. Grx3 is found in the cytosol, nucleus and extracellular. It was identified in a yeast-two hybrid screen as a potential binding partner of protein kinase C-θ. The overexpression of Grx3 leads to attenuated activation of JNK and the transcription factor NFκB (Witte et al. 2000). In addition, Grx3 affects immune signaling (Kato et al. 2008), (Babichev and Isakov, 2001), protects against cardiac hypertrophy (Jeong et al. 2006), (Jeong et al. 2008), (Cha et al. 2008) and was the second mammalian glutaredoxin identified as FeS protein (Haunhorst et al. 2010). Knock-out of Grx3 in mice is embryonically lethal between days 12.5 and 14, with embryos not showing any defects in organogenesis (Cha et al. 2008). The monothiol Grx5 shares the active site motif of Grx3 and has a mitochondrial translocation signal. Grx5 is located in mitochondria and so far, no disulfide reductase activity was observed. Up to now, there are no reports on knock-out mice, but loss of Grx5 in zebrafish leads to the so called "*shiraz*" phenotype which is characterised by hypochromic anemia due to a disruption in iron homeostasis (Wingert et al. 2005). A similar phenotype was observed in a patient with reduced Grx5 levels (Camaschella et al. 2007).

Figure 7 illustrates the compartmentalisation of a cell into nucleus, cytosol, mitochondrium and peroxisome, as well as the thioredoxin family proteins and related proteins described above. Each protein is depicted, showing the individual Grx- and/or Trx-domain(s) as well as the cysteine-containing active site motif(s). The localisation, as well as the electron flow, of the Trx system (NADPH, TrxR, Trx, Txnip and Nrx), the Grx system (NADPH, GR, GSH, Grx and γGCS) and the peroxiredoxins is shown. The discovered translocation and secretion of particular proteins is also included.

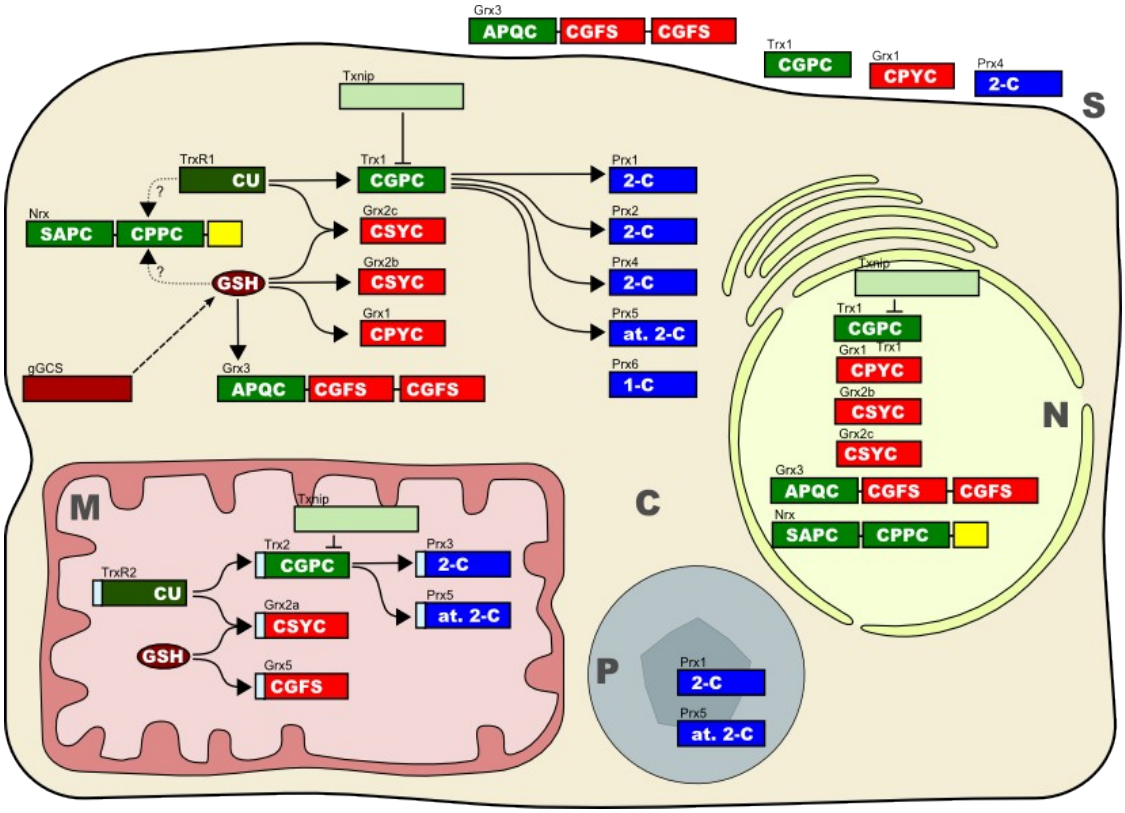

Figure 7: Trx family proteins in different cellular compartments. Trxs are depicted in green, Prxs in blue, Grxs in red. Protein domains and active site motifs of every protein are indicated. C=cytosol, M=mitochondrium, N=nucleus, P=peroxisome, S=subcellular space (adapted from Godoy et al. 2011a).

1.3 Hypoxia-related disorders

The term hypoxia describes a condition of insufficient oxygen supply, either to the whole body or to single organs or tissues. This condition has been implicated in different disorders. However, O_2 concentrations are essential in regulating embryonic development as well, i.e. some cell types proliferate at low O_2 concentrations, whereas higher oxygen levels promote cell differentiation (Covello et al. 2006), (Studer et al. 2000). The same is valid for placental development (Adelman et al. 2000). Pathologies triggered by a hypoxic insult usually result from a blockage or reduction in blood flow leading not only to the absence of oxygen, but also ATP and other substrates and nutrients. Reasons can be a cerebral stroke or a heart attack, but it can also occur during organ transplantation. Most of the tissue and cellular damage occurs when the oxygen returns to the affected tissue. This reoxygenation phase leads to molecular and cellular alterations such as morphological changes, loss of cell polarity, elevated release of neurotransmitters in the brain, defective osmoregulation and inhibition of protein synthesis (Johnston et al. 2001). Many cancer cell lines have been shown to grow under

low oxygen levels, with tumor hypoxia being a criterium for aggressiveness of the disease and resistance to radiation therapy (Harris, 2002). Physiological and pathological processes are generally dependent on the hypoxia-inducible factors (HIF) 1 and 2, transcription factors regulating the expression of at least 180 genes. Targets include erythropoietin, increasing the production of erythrocytes, vascular endothelial growth factor, elevating vascularization of affected tissues, as well as metabolic proteins (Adelman et al. 1999), (Semenza, 2000).

1.3.1 Perinatal asphyxia

Perinatal asphyxia is a condition where a newborn suffers from oxygen deprivation caused for instance by a drop in maternal blood pressure, placental abruption, umbilical cord compression during child birth or the inability to breath after birth. Worldwide, perinatal asphyxia constitutes with 920,000 neonatal deaths annually a major cause of death. It is furthermore associated with 1.1 million intrapartum stillbirths (Lawn et al. 2005). 25 % of the surviving infants suffer from permanent neurological deficits such as spasticity, epilepsy, mental retardation and vision and hearing impairments, as well as learning deficits (Vannucci and Perlman, 1997), (Gunn, 2000), (Shankaran, 2009). So far, no effective therapeutic approaches to prevent or treat the damage caused by the hypoxia/ischemia insult are available. However, since the first report in 1964 by Miller and coworkers, many publications have described hypothermia as an effective treatment for hypoxia-induced damages to the brain (Miller et al. 1964), (Capani et al. 2003), (Webster et al. 2009). Golgi type I neurons in cerebral cortex, hippocampus, neostriatum and cerebellum (Capani et al. 2009), (Kirino et al. 1992) are most affected. The mechanisms leading to neuronal cell death have up to now, not been understood. However, many immediate as well as long term alterations have been described such as overrelease of excitatory amino acids, overactivation of glutamate receptors placed on the post-synaptic density (Choi et al. 1989), (Choi et al. 1995) NO and ROS (Capani et al. 1997), (Capani et al. 2001) and reactive astrogliosis, high immunoreactivity of high weight neurofilaments (Saraceno et al. 2010) and increased levels of ubi-proteins and free ubiquitin at the postsynaptic density in the neostriatum (Capani et al. 2009).

1.3.2 Renal ischemia/reperfusion injury

Renal ischemia/reperfusion (I/R) injury constitutes a major clinical problem, for which no treatment is available yet. It is the leading cause of acute renal failure and may lead to the development of chronic kidney diseases (Weight et al. 1996), (Sutton et al. 2002). It is caused by a temporary blockage or decrease in the blood supply of the kidney, caused by a functional constriction of blood vessels or after an organ transplantation. The ischemic period is followed by the restoration of the blood flow, the reperfusion, which itself leads to additional cellular damage (Mejía-Vilet et al. 2007). Tubular cells are most susceptible to an ischemic insult and undergo necrosis and apoptosis due to complex molecular and structural alterations. During the reperfusion period a strong immune response is initiated. Various cytokines, as well as endothelial adhesion molecules are upregulated. ROS, produced by activated leukocytes and NO, produced by inducible nitric oxide synthetase, harm different cellular structures and molecules, leading to a dismorphic cell shape and loss of cell polarity. In addition, activation of mitogen-activated protein kinases, leads to increased cell death (Hutchens et al. 2008). Trx1 was analysed in a mouse model for renal I/R injury. Kasuno *et al.* demonstrated that the expression of Trx1 was increased in the medullary thick ascending limbs (mTALs) after an ischemic insult, while the protein was secreted from cortical proximal tubuli into the urine. Moreover, Trx1 overexpressing mice were less susceptible to the I/R injury of the kidney, especially to the mTALs (Kasuno et al. 2003).

1.3.3 Transplantation of pancreatic β-cells – therapeutic approach for diabetes mellitus

Diabetes mellitus is a chronic disease, which constitutes an emerging health problem with 220 million cases worldwide according to the WHO in 2010. Type I diabetes is an autoimmune disease. The immune system attacks the β-cells of the pancreas that synthesize and release insulin into the portal vein. Consequently, not enough insulin is produced, a hormone needed for the regulation and cellular uptake of glucose, as well as glucose storage as glycogen. Type II diabetes results from insulin resistance, i.e. the insulin is not efficiently used and due to constant glucose overflow pancreatic β-cells will gradually die from metabolic stress (Liu et al. 2000). Symptoms of the disease are glycaemia, polyuria, polydipsia, constant hunger, weight loss, vision changes and fatigue. Long-term effects include damage of heart, kidneys, eyes, blood vessels and neurons, e.g. 50 % of patients die of a cardiovascular disease. Treatments include a

change in diet and the administration of insulin. Since the 1960s, surgical treatment for diabetes mellitus has improved and constitutes an alternative to permanent insulin injections (Jahansouz et al. 2011). The transplantation of pancreatic islets is a promising strategy in order to restore insulin secretion. However, ROS generated during islet isolation and transplantation, as well as general inflammation reactions may prevent long-term survival and regeneration of β-cells (Chou and Sytwu, 2009). It was shown that Txnip is induced by glucose and is upregulated in diabetic animal models and patients, inhibiting Trx activity (Schulze et al. 2004). Overexpression of Trx1 in models for type I and type II diabetes minimized cellular damage and improved the survival of β-cells (Hotta et al. 1998), (Yamamoto et al. 2008). Moreover, it reduced and prevented associated conditions such as diabetic embryopathy (Kamimoto et al. 2010) and diabetic osteopenia (Hamada et al. 2009). Wolf and collegues stated that downregulation of Prx3 in rat insulinoma cells led to insufficient insulin secretion, while overexpression protected the cells against various agents, including H_2O_2, NO, proinflammatory cytokines and streptozotocin, a β-cell toxin (Wolf et al. 2010). In addition, Chou and Sytwu transfected healthy mouse islets with a lentivirus vector coding for Trx1, before transplantation into diabetic NOD mice. Trx1 overexpressing islets were resistant against inflammatory processes and significantly prolonged islet survival after transplantation, without showing any differences in the glucose-dependent insulin secretion *in vitro* (Chou and Sytwu, 2009).

1.4 Aims of the study

The proteins of the thioredoxin family are major players in redox regulation as they control the redox state of protein thiols and the levels of the signal molecule hydrogen peroxide. Some of the thioredoxin family members have significant effects on the cell cycle. Our overall aim was to understand the role of these redoxins under physiological as well as pathological conditions, i.e. under oxygen deprivation using different cell culture and animal models. We were especially interested in determining and defining the role of the Trx family proteins in terms of controling the fate of cells. We furthermore wanted to gain more information on single proteins of the Trx family, looking at protein interactions, target proteins and specific functions. The specific aims of this thesis were:

- to create new tools for the analysis of intracellular levels of distinct redoxins, namely the generation of specific polyclonal antibodies and a Grx2-specific sandwich ELISA.
- to establish the use of siRNAs and create overexpressing plasmids for selected redoxins in order to manipulate protein levels in different cell lines.
- to answer the question if Grx2 can donate electrons to the mitochondrial Prx3 *in vivo*.
- to determine if other members of the thioredoxin family of proteins, except Grx5, are involved in iron homeostasis and FeS cluster biosynthesis.
- to analyse the distribution of Trx family proteins in the central nervous system (CNS) of the rat.
- to examine the expression level and distribution/ translocation of the Trx family proteins upon oxygen deprivation in distinct cell lines and animal models for perinatal asphyxia, renal ischemia-reperfusion injury and the transplantation of pancreatic β-cells, as a therapeutic approach for diabetes.
- to explore if the overexpression of redoxins in distinct cell types can alter the fate of a cell i.e. protecting cells against cell death, induced by a hypoxic insult.

2. Materials and Methods

2.1 Materials

2.1.1 Instruments

Äkta Prime FPLC system	GE Healthcare (Uppsala, Sweden)
Agarose gel electrophoresis system Varia 1	Roth (Karlsruhe, Germany)
Autoclave	Systec (Wettenberg, Germany)
Camera Chemocam HR16	Intas (Göttingen, Germany)
Centrifuges	
Avanti J-20 XP	Beckmann (Munich, Germany)
Table centrifuge Biofuge fresco/pico	Heraeus (Hanau, Germany)
Easyject Plus Equibio Electroporator	Equibio (Willstätt, Germany)
Electrophoresis power supply ECPS3000/150	GE Healthcare (Uppsala, Sweden)
Fermenter R´ALF PLUS SOLO, 6,7 l	Bioengineering (Wald, Switzerland)
Gel Imager	Kodak (Stuttgart, Germany)
Heating Block Epptherm	Liebisch (Bielefeld, Germany)
Incubator	Heraeus (Hanau, Germany)
Liquid homogenizer	Microlor (Villers-Les-Nancy, France)
Magnetic Stirrer MR3000	Heidolph (Kehlheim, Germany)
Microscopes	
Confocal microscope LCS SP2	Leica (Wetzlar, Germany)
Light microscope Leica Diaplan	Leica (Wetzlar, Germany)
Microscope Camera Micro Publisher	Qimaging (Surrey BC, Canada)
PH meter	G-Tech Instruments (Hsinchu, Taiwan)
Photometer Specord S300 UV Vis	Analytic Jena (Jena, Germany)
Scintillation counter	Beckman Coulter (USA)
Shaking incubator Innova 4300	New Brunswick Scientific (New Jersey, USA)

Sterile Benches	Integra Biosc. (Fernwald, Germany)
Thermocycler T3000	Biometra (Göttingen, Germany)
Vortex genie II	Scientific Industries Inc. (USA)
Water bath Lauda M3	Lauda Dr. R. Wobster GmbH
Xcell II™ Blot Module	Invitrogen (Karlsruhe, Germany)
Xcell SureLock® Mini Cell, PAGE Chamber	Invitrogen (Karlsruhe, Germany)
96-well plate reader Infinite M200	Tecan (Crailsheim, Germany)

2.1.2 Chemicals

Chemicals used in this study were purchased in analytic grade or better at the following companies: Carl-Roth (Karlsruhe, Germany), Fermentas (St. Leon-Rot, Germany), Gibco Life Technology (Karlsruhe, Germany), Merck (Darmstadt, Germany), Roche Diagnostics GmbH (Mannheim, Germany), Sigma-Aldrich (Steinheim, Germany). Consumable supplies like pipette tips, Falcon tubes and cell culture bottles were from Carl-Roth (Karlsruhe, Germany), Greiner (Frickenhausen, Germany), Kobe (Marburg, Germany) and Sarstedt (Nuembrecht, Germany). Various Kits used in this study were bought from BioRad (Hercules, USA), Fermentas (St. Leon-Rot, Germany), Promega (Mannheim, Germany), Quiagen (Hilden, Germany), and Roche Diagnostics GmbH (Mannheim, Germany). Columns for protein purification and nitrocellulose membranes were purchased from GE Healthcare (Uppsala, Sweden); SDS gradient gels from Thermoscientific Fisher (Schwerte, Germany). Compounds such as antibiotics, buffer, cell media and supplements, enzymes, marker for DNA and proteins were from Fermentas (St. Leon-Rot, Germany), Finnzymes (Espoo, Finnland), Invitrogen (Groningen, Germany), New England Biolabs (Frankfurt, Germany) and PAA (Cölbe, Germany).

2.1.3 Antibodies

In order to detect specific proteins in cells, cell lysates or mouse/rat tissues, we have used specific polyclonal antibodies.

Most of these antibodies were generated by the AG Lillig as described in 2.2.3.2, part of them within this study (compare to 3.1.1), or were purchased from companies stated in Table 1.

Name	*Source*	*Western Blot*	*IHC*	*Origin/ Company*
Actin	Mouse	1 to 5000	-	Santa Cruz Biotechnology (USA)
DNP	Rabbit	1 to 1000	-	Acria Antibodies (Hiddenhausen, Germany)
ERK	Rabbit	1 to 1000	-	Cell signaling Technology (USA)
ERK (ph)	Rabbit	1 to 1000	-	Cell signaling Technology (USA)
Ferritin	Rabbit	1 to 1000	-	MP Biomedicals (USA)
GAPDH	Rabbit	1 to 5000	-	Sigma Aldrich (Steinheim, Germany)
GPAT	Rabbit	1 to 7500	-	Kind gift of AG Lill (Marburg, Germany)
Grx1	Rabbit	1 to 200	1 to 200	Santa Cruz Biotechnology (USA)
Grx2	Rabbit	ELISA	1 to 500	Serum and affinity purified, AG Lillig
Grx3	Rabbit	1 to 1000	1 to 25	Serum and affinity purified, AG Lillig
Grx5	Rabbit	1 to 1000	1 to 1000	Serum, AG Lillig (Marburg, Germany)
GSH	Mouse	1 to 1000	-	Virogen (Boston, USA)
IRP1	Rabbit	1 to 1000	-	Serum, AG Lillig
JNK	Rabbit	1 to 1000	-	Cell signaling Technology (USA)
JNK (ph)	Rabbit	1 to 1000	-	Cell signaling Technology (USA)
Mouse HRP	Goat	1 to 5000	-	BioRad (Hercules, USA)
mtAco3	Rabbit	1 to 3000	-	Kind gift of AG Lill (Marburg, Germany)
mTrx1	Rabbit	1 to 1000	1 to 1000	Serum, AG Lillig
Nrx	Rabbit	1 to 1000	-	Kind gift of AG Zimmermann (Homburg, Germany)
p38	Rabbit	1 to 1000	-	Cell signaling Technology (USA)
p38 (ph)	Rabbit	1 to 1000	-	Cell signaling Technology (USA)
Prx1*	Rabbit	1 to 1000	1 to 500	Serum, AG Lillig
Prx2*	Rabbit	1 to 1000	1 to 200	Santa Cruz Biotechnology and serum, AG Lillig
Prx3	Rabbit	1 to 1000	1 to 500	Serum, AG Lillig
Prx4*	Rabbit	1 to 2000	1 to 500	Santa Cruz Biotechnology (USA)
Prx5	Rabbit	1 to 1000	1 to 500	Serum, AG Lillig
Prx6*	Rabbit	1 to 1000	1 to 500	Serum, AG Lillig
Rabbit HRP	Goat	1 to 5000	-	BioRad (Hercules, USA)
rTrxR1	Rabbit	1 to 1000	1 to 100	Serum and affinity purified, AG Lillig
TfR	Mouse	1 to 3000	-	Zymed (San Franscisco, USA)
Trx1	Rabbit	1 to 1000	-	Serum, AG Lillig
Trx2	Rabbit	1 to 1000	1 to 1000	Serum, AG Lillig
TrxR1	Rabbit	1 to 1000	-	Serum, AG Lillig
TrxR2	Rabbit	1 to 200	1 to 200	Santa Cruz Biotechnology (USA)
Tubulin	Mouse	1 to 5000	-	Sigma-Aldrich (Steinheim, Germany)
Txnip	Rabbit	1 to 200	1 to 200	Sigma-Aldrich (Steinheim, Germany)
γGCS	Rabbit	1 to 200	1 to 100	Santa Cruz Biotechnology (USA)

Table 1: Sera and purchased antibodies used within this study. Sera were generated within this study (*); (ph=phosphorylated). Dilutions for Western Blot and Immunohistochemistry (IHC) are enlisted.

2.1.4 Oligonucleotides

Oligonucleotides used in this study were designed according to the following rules: The length was around 16-30 bp and the guanine/cytosine content was around 50 %. Primer pairs were designed with melting temperatures (T_m) as similar as possible. T_m was calculated using Promegas biomath calculators (www.promega.com/biomath/Default.htm). Oligonucleotides were ordered from DNA Technology GmbH (Aarhus, Denmark), used in stock concentrations of 100 pmol/µl and stored at -20 °C. All primer pairs are summarized in Table 2 and Table 3, stating their sequence, amplificates, endonucleases as well as the target plasmid.

Name: amplificate	***Endo-nucleases***	***Sequence***	***Target plasmid***
PREMH1: hPrx1, Fw	NdeI	**CATATG**tcttcaggaaatgctaaaattgg	pet15b
PREMH2: hPrx1, Rv	BamHI	**GGATCC**tcacttctgcttggagaaatattc	
PREMH3: hPrx2, Fw	NdeI	**CATATG**gcctccggtaacgcg	pet15b
PREMH4: hPrx2, Rv	BamHI	**GGATCC**ctaattgtgtttggagaaatattccttg	
PREMH7: Prx6, Fw	NdeI	**CATATG**cccggaggtctgc	pet15b
PREMH8: Prx6, Rv	BglII	**AGATCT**ttaaggctggggtgtgtag	
PREMH9: hPrx3, Fw	NheI	**GCTAGC**atggcggctgctgtaggac	pExpress
PREMH10: hPrx3, Rv	BglII	**AGATCT**ctactgatttaccttctgaaagtactc	
PREMH11: hPrx5, Fw	NheI	**GCTAGC**atggccccaatcaaggtggg	pExpress
PREMH12: hPrx5, Rv	BglII	**AGATCT**tcagagctgtgagatgatattggg	
PREMH13: hPrx4, Fw	NdeI	**CATATG**gaggcgctgc	pet15b
PREMH14: hPrx4, Rv	BamHI	**GGATCC**ggatcctcaattcagtttatcgaaatacttc	
PREMH15: hPrx1, Fw	NheI	**GCTAGC**atgtcttcaggaaatgctaaaattgg	pExpress
PREMH16: hPrx1, Rv	BglII	**AGATCT**tcacttctgcttggagaaatattc	
PREMH17: hPrx2, Fw	NheI	**GCTAGC**atggcctccggtaacgcg	pExpress
PREMH18: hPrx2, Rv	BglII	**AGATCT**ctaattgtgtttggagaaatattccttg	
PREMH19: hPrx4, Fw	NheI	**GCTAGC**atggaggcgctgc	pExpress
PREMH20: hPrx4, Rv	BglII	**AGATCT**ggatcctcaattcagtttatcgaaatacttc	
PREMH21: hPrx6, Fw	NheI	**GCTAGC**atgcccggaggtctgc	pExpress
PREMH12: hPrx6, Rv	BglII	**AGATCT**tcagagctgtgagatgatattggg	

Table 2: Oligonucleotides for cloning peroxiredoxins. Prxs 1, 2, 4 and 6 were cloned in pet15b for protein production and generation of antibodies. Prxs 1, 2, 3, 4, 5 and 6 were cloned in pExpress for overexpression in human cells. Primer sequences include restriction sides for the indicated restriction enzymes, which are written in cover letters.

Name	*Target*	*Mutation*	*Purpose*
PRVCM001 PRVCM001R	Grx2 in pEGFP-N1	S74P	Active site mutation; protein cannot hold an FeS cluster.
PRVCM002 PRVCM002R	Grx2 in pEGFP-N1	T95R	Mutation leading to an attenuated binding of GSH and thus of the FeS cluster.

Table 3: Oligonucleotides for mutagenesis of human glutaredoxin 2 in pEGFP-N1.

2.1.5 siRNAs

Specific siRNAs were used to silence the expression of different redoxins in cells. Unspecific scrambled siRNA was used as control. Double-stranded RNAs were designed with sequences complementary to the gene of interest and were purchased from Ambion (Austin, USA) or Eurogentec (Seraing, Belgium). They were thawn, aliquoted and stored at – 80 °C. All established and used siRNAs are listed in Table 4.

Name	*Target protein*	*Sequences (sense, antisense)*
Scram*	none; control	CAUUCACUCAGGUCAUCA, CUGAUGACCUGAGUGAAU
Grx1si*	hGrx1	GGGCUUCUGGAAUUUGUCG, CGACAAAUUCCAGAAGCCC
Grx2si*	hGrx2	GGUGCAACUGACACUCAU; UAUGAGUGUCAGUUGCAC
Grx3si	hGrx3	GCCUAUUCCAGUUGGCCUA; UAGGCCAACUGGAAUAGGC
Trx1si*	hTrx1	GUAGAUGUGGAUGACUGUC, GACAGUCAUCCACAUCUAC
Trx2si*	hTrx2	GGAUCUCCUUGACAACCU; AAGGUUGUCAAGGAGAUC

Table 4: SiRNAs used to silence the expression of redoxins in human cell lines. SiRNAs labeled with an asterisk were established within this study.

2.1.6 Computer based data mining

2.1.6.1 Analysis of Western Blots

Image J was used to analyse and quantify the protein bands on Western Blots.

2.1.6.2 Figures

Figures and diagrams were created using the GNU Image Manipulation Program, InkScape or Xmgrace.

2.1.7 Strains and plasmids

2.1.7.1 Bacteria

E. coli XL-1 blue mrf`: Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 *end*A1 *sup*E44 *thi*-1 recA1 *gyr*A1 *gyr*A96 *rel*A1 *lac* F`[*pro*AB*lac*I^{q}ZΔM15 Tn10(Tetr)]c Stratagene (tetracycline resistance)

E. coli BL21(DE3)pRIL: *hasdS gal* (λI*ts*857 *ind* 1 *S*am7 *nin*5 lac UV5-T7 gene 1) Stratagene, (chloramphenicol resistance)

2.1.7.2 Cell lines

HeLa cells are derived from a cervical carcinoma of the patient Henrietta Lacks in the 1950th. They constitute an established cell line of human epithelial cells and the most commonly used human cell line. HeLa cells were cultivated in low glucose DMEM supplemented with 10 % heat inactivated fetal calf serum (FCS) and 100 U/ml penicillin and streptomycin.

HEK293 cells are derived from human kidney cells, obtained from a healthy aborted fetus. The cells were immortalised using adenovirus 5 DNA. Cells were cultivated in low glucose DMEM supplemented with 10 % heat inactivated FCS and 100 U/ml penicillin and streptomycin.

INS1 cells are derived from rat pancreatic β-cells. Cells were cultivated in RPMI medium supplemented with 10 % heat inactivated FCS, 100 U/ml penicillin and streptomycin, 10 mM HEPES, 1 mM sodium pyruvate and 50 µM mercaptoethanol.

MIN6 cells are derived from mouse pancreatic β-cells. They constitute a highly differentiated and glucose responsive cell line. MIN6 cells were cultivated in high glucose DMEM, supplemented with 20 % heat inactivated FCS, 100 U/ml penicillin and streptomycin, 1 mM sodium pyruvate and 71 µM mercaptoethanol.

Primary islet cells are healthy, non immortalised cells, derived from pigs. These cells do not divide. They were kept in CMRL 1066 medium, supplemented with 20 % heat-inactivated FCS, 100 U/ml penicillin and streptomycin, 2.5 mM glutamine, 1 mM sodium pyruvate, 7 % glucose, 0.2 % sodium bicarbonate, ciprobay 200.

2.1.7.3 Plasmids

pGEM-T Vector (Promega):

The pGEM-T vector was used for direct cloning of PCR products. It contains single 3`thymine overhangs at the insertion site, carries a T7 promoter and ampicillin resistance (Figure 8).

(vector cards were derived from the manufacturors)

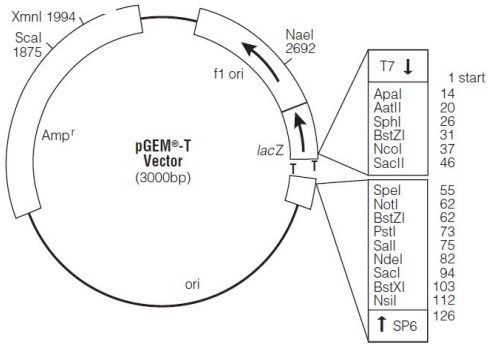

Figure 8: pGEM-T vector information.

pet15b (Novagen):

The pet15b vector was used for protein expression. It carries a T7 promoter, a His Tag sequence and an ampicillin resistance (Figure 9).

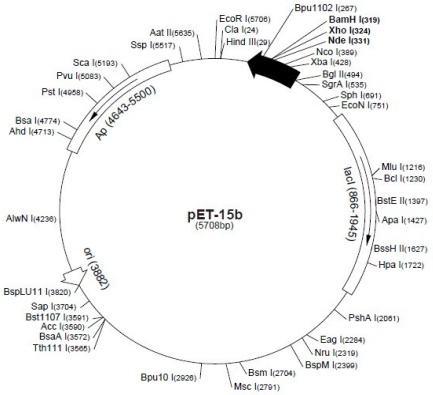

Figure 9: pet15b vector information.

pExpress (Arakawa et al. 2001):

The plasmid was used for the general overexpression of proteins in cells. It uses two promoters, allowing transcription in *E. coli* and in eukaryotic cells. pExpress carries an ampicillin resistance (Figure 10).

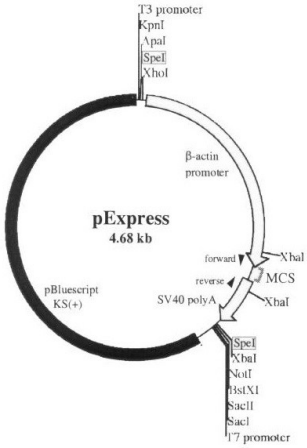

Figure 10: pExpress vector information.

pEGFP-N1 (Clontech, Germany):

The plasmid was used for protein over-expression in cells with attention to protein localization. pEGFP-N1 contains a fluorescent protein (Excitation: 488nm; Emission: 507 nm) at the N-terminus, which is fused to the protein of interest. It carries two promoters for transcription in *E. coli* and eukaryotic cells and a kanamycin resistance (Figure 11).

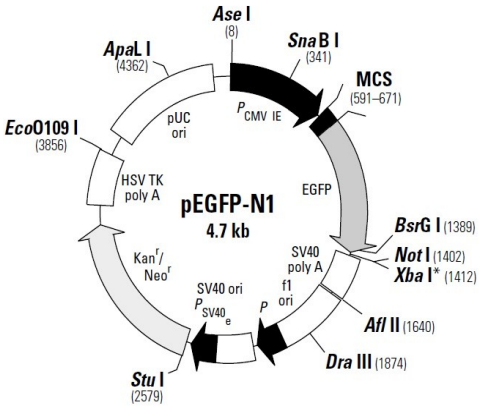

Figure 11: pEGFP-N1 vector information.

2.1.8 Animal models

All experiments were performed in agreement with the legislation for the ethical use of laboratory animals in each country.

2.1.8.1 Rat model for perinatal asphyxia

Pregnant female rats were kept in individual cages with controlled 12:12 h light/dark cycle (temperature: circa 21 °C; humidity: circa 65 %). Animals were divided into three groups: 1. control group, which gave birth to control pups; 2. surrogate mothers; 3. animals assigned to the perinatal asphyxia procedure. Fullterm pregnant rats were rendered unconscious by CO_2 inhalation (Dorfman et al. 2006) and were rapidly decapitated. An abdominal incision was made and the uterus horns were isolated. The pups were put into a 37 °C warm waterbath for maximum 19 min. Times exceeding 19 minutes lead to a very low survival rate of the animals. Subsequently the uterus horns were opened and the pups were cleaned from amniotic fluid. The pups breathing was stimulated for a few minutes by performing tactile intermittent until the pups were breathing regularly. The umbilical cord was cut and the pups were observed for approx. 1 hour, while keeping them warm using a heating lamp. The pups were marked and mixed with the litters of the surrogate mothers as soon as their physiological conditions improved (Figure 12). Only male animals were used for later analysis.

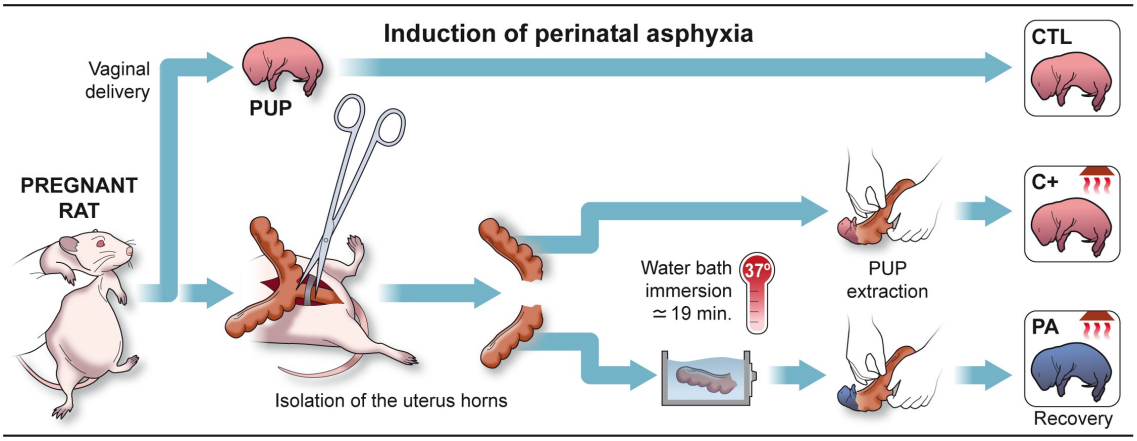

Figure 12: Rat model for induction of perinatal asphyxia. Fullterm pregnant rats were killed by CO_2 inhalation, the uterus horns were isolated and incubated in a 37 °C waterbath for 19 min. Pubs were extracted, cleaned from uterus tissue and breathing was stimulated. Control pubs were isolated in the same way, without exposing them to the asphyxic condition. All pubs were labeled and were mixed with the litters, vaginal delivered by the surrogate mothers (provided by Prof. Dr. Francisco Capani).

2.1.8.2 Mouse model for ischemia/reperfusion injury

Male (10-12 weeks old) C57BL/6J mice (Charles River, Germany) were narcotised with 100 mg/kg ketamine (Pfizer, Karlsruhe, Germany) and 5 mg/kg xylazine (Rompun, Bayer Leverkusen, Germany). To ensure a constant body temperature of 37 °C the mice were operated on a heat plate. An incision was made on the right side, exposing the right kidney. The kidney pedicle was clamped. The kidney was kept warm and moist during the whole procedure. After 30 min the clamp was removed, the restoration of the blood flow was observed and the incision was sutured. In control animals or so called sham operated mice the incision was made and the kidney was exposed for 30 min, but it was not clamped. The animals were kept in cages and blood and urine samples were taken after 24 h reperfusion time. The mice were sacrificed by carbon dioxide intoxication followed by cervical dislocation and the ischemic, as well as the contralateral kidney were removed.

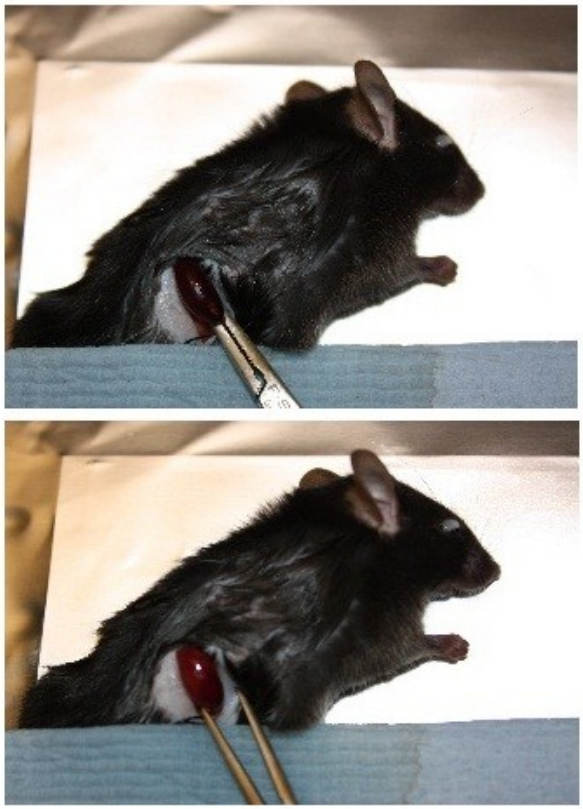

Figure 13: Mouse model for renal ischemia/ reperfusion injury. Male C57BL/6J mice were anaesthetised, an incision was made and the pedicle of the right kidney was clamped, restricting the blood flow. After 30 min the clamp was removed and the blood flow was restored (provided by Dr. José Godoy).

2.1.8.3 Mouse model for diabetes mellitus

In the AG Linn (Justus-Liebig Universität, Faculty of Medicine, Gießen) mice models have been established, which mimic different aspects of diabetes. One model for type-I diabetes is the streptozotocin-induced diabetes. Streptozotocin is a β-cell toxin. C57BL/6 mice were injected intra-peritoneal with 200 mg/kg streptozotocin. Control animals received citrate buffer instead. Within the following three days the blood sugar levels were determined; if the blood sugar level was higher than 200 mg/dl for more

than 2 days the animal was considered diabetic. To mimic the conditions during a transplantation of pancreatic islet cells, islets from healthy donor mice were isolated and injected into the portal vein of a diabetic mouse.

2.1.8.3.1 Isolation of pancreatic islets

Hank solution: 9.5 g/l Hank´s balanced salts, 0.35 g/l $NaHCO_3$, penicillin 0.6 g/l; Collagenase solution: 30 mg collagenase in 12 ml Hank solution

Mice were narcotised using 0.3 ml/20 g avertin and sacrificed by cervical dislocation. The pancreas was removed and 3 ml collagenase solution were injected into the ductus choledochus/pancreaticus. The pancreas was incubated with the rest of the collagenase solution for 10 min at 37 °C. The digestion was improved mechanically by vortexing every 2-3 min. The tissue was put on ice and cold Hank solution was added. After centrifuging for 3 min at 1500 rpm the supernatant was mixed with medium supplemented with 10 % FCS. The cells were transferred into a petri dish. Single islets were hand-picked, transferred into another petri dish and were cultivated over night at 37 °C in a humidified atmosphere containing 5 % CO_2 and 20 % O_2.

2.2 Methods

2.2.1 Molecular biological methods

2.2.1.1 Standard techniques

Agarose-gelelectrophoresis, ligation, DNA digestion with endonucleases and extraction of DNA were performed according to standard protocols. Ligation in pGEM-T was performed according to the manufacturer´s protocol (Promega). For gel elution and DNA extraction the Nucleo-Spin-Extract Kit II was used.

2.2.1.2 Polymerase chain reaction

The polymerase chain reaction (PCR) is a method to amplify DNA. The reaction takes place in a thermocycler in the presence of: 2-20 ng template DNA, 20 pmol/µl of a specific primer pair, 0.2 mM dNTPs, 1x reaction buffer containing 1.5 mM $MgCl_2$ (Finnzymes, Espoo, Finland) and 0.5-2.5 U DyNAzyme EXT. DNA was denatured for

1 min at 94 °C. 34 cycles of denaturing at 92 °C for 30 sec; annealing at a primer specific temperature for 45 sec; elongation at 72 °C for 45 sec, followed. After a final elongation step at 72 °C for 10 min the PCR product was loaded on a 1 % agarose gel and was extracted using the Nucleo-Spin-Extract Kit II.

2.2.1.2.1 Rolling circle PCR

The rolling circle polymerase chain reaction or mutagenesis PCR is similar to the conventional PCR reaction. Special primers, containing a mismatch in the middle, leading to a point mutation, are used and an extended elongation time is needed for amplifying the whole vector. DNA was denatured for 1 min at 90 °C. 15 cycles of denaturing at 90 °C for 30 sec; annealing at a primer specific temperature for 1 min; elongation at 68 °C for a plasmid size specific duration (1000 bp correlate to 1 min), followed. The amplified DNA was digested with the restriction enzyme *DpnI*, which cuts only methylated template DNA.

2.2.1.3 Generation and transformation of competent cells

RF1 buffer: 100 mM RbCl, 50 mM $MgCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$, 15 % glycerol, pH 5.8; RF2 buffer: 10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15 % glycerol, pH 6.8

Competent bacteria were generated based on the rubidiumchloride method. *E. coli* cells were grown until they had reached the exponential phase (OD_{600} of 0.6). Cells were incubated on ice for 15 min and were spun down in DNA free tubes in a centrifuge at 1000xg for another 15 min. The pellet was resuspended in 1/3 volume of RF1 buffer without vortexing and was incubated on ice for 15 min. The bacteria were centrifuged at 1000xg for 15 min and were resuspended in 1/12.5 volumes of RF2 buffer. After another incubation on ice for 15 minutes, the cells were aliquoted, frozen in liquid nitrogen and stored at -80 °C. 25 ng of DNA and 200 µl competent cells were incubated on ice for 20 min. A heat shock was performed at 42 °C for 90 seconds in a heating block. After an incubation on ice for 5 min, the sample was incubated with 800 µl LB medium at 37 °C for 45 min. Cells were spun down in a centrifuge for 2 min at 8000 rpm, the pellet was resuspended in 50 µl LB medium and bacteria were transferred to a LB plate, containing special antibiotics, which was incubated over night at 37 °C.

2.2.1.4 Sequencing

All plasmids generated in this study were send to Seqlab (Göttingen, Germany) for sequencing. Ferograms were analysed using Finch TV, sequences using NCBI BLAST and ExPASy.

2.2.2. Cell biological methods

2.2.2.1 Cell cultivation and cell splitting

All cell lines used in this study were cultured at 37 °C in a humidified atmosphere containing 5 % CO_2 and 20 % O_2, if not stated otherwise. Cell lines and the appropriate medium are mentioned in 2.1.7.2. In general, the medium was changed every 3 days. When cells were confluent, they were splitted. Old medium was removed, cells were washed once with PBS and were incubated a few minutes with trypsin at 37 °C. By adding fresh medium the reaction was stopped. Cells were splitted in a ratio between 1:3 – 1:10. MIN6 cells were centrifuged for 5 min at 700 rpm to get rid of the trypsin, before seeding them out in fresh medium in a ratio of 1:2 or 1:3.

2.2.2.2 Freezing and thawing of cells

Cells were harvested and resuspended in medium supplemented with FCS and 10 % DMSO. Cells were frozen in an isopropanol chamber at -80 °C. After 48 h, cells were transferred in liquid nitrogen. Cells were thawn in 5 ml fresh medium. To remove DMSO, cells were centrifuged for 5 min at 700 rpm. Cells were seeded out in fresh medium.

2.2.2.3 Cell counting

Cells were counted using a Neubauer counting chamber according to the provider. 20 µl of the cell suspension were added to the chamber. Cells were counted using a light microscope.

2.2.2.4 Cell lysis and preparation of crude cell extract

NP40-lysis buffer: 10 mM Tris/HCl, 0.1 % NP40, 10 mM NaCl, 3 mM $MgCl_2$, protease and phosphatase inhibitors, pH 7.4; NEM-lysis buffer: 40 mM HEPES, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 100 mM NEM, protease and phosphatase inhibitors, pH 7.4

Cells were harvested, washed with PBS and resuspended in lysis buffer and incubated at RT for 15 min, before freezing the cells in liquid nitrogen. In the case of NEM-lysis, 2 % CHAPS were added right before freezing. Samples were stored at -20 °C. Samples were thawn, centrifuged at 4 °C for 5 min at 13000 rpm and the supernatant and the crude cell extract were transferred into new tubes.

2.2.2.5 Cell fractionation

Mitobuffer: 5 mM Tris/HCl, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$, protease inhibitors, pH 7.4; Digitonin buffer: Mitobuffer containing 0.008 % digitonin

Cells were harvested, washed once in PBS and were lysed in ice cold digitonin buffer (2 ml for 3.5 Mio cells). After an incubation on ice for 10 min one third was transferred to a new tube and was frozen in liquid nitrogen as the total cell lysate. The rest was centrifuged at 4 °C for 10 min at 13000 rpm. The supernatant was transferred to a new tube and was frozen as the cytosolic fraction. The pellet was washed once with 1 ml Mitobuffer and was resuspended in 200 µl of Mitobuffer. This part was shock frozen as mitochondria-rich fraction. Samples were stored at -80 °C.

2.2.2.6 Cell transfection

2.2.2.6.1 Electroporation

Electroporation buffer: 21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM D-glucose, pH 7.15

3.5 Mio HeLa cells were harvested, washed once in PBS and were resuspended in 600 µl electroporation buffer. 15 µg plasmid DNA or siRNA were mixed thoroughly with the cells and were transfected in an electroporation cuvette using 250 mV and 1500 µF for 25-30 ms. 600 µl FCS were added immediately to the cells before seeding them out in 1:5 conditioned medium.

2.2.2.6.2 Chemical transfection using lipofectamin

Different amounts of MIN6 cells were seeded out in different well plates (see Table 5). DNA and lipofectamin were incubated in 200 µl antibiotic free medium separately for 5 min, before mixing them. After a 30 min incubation at RT the DNA-Lipofectamin

complexes were transferred to the cells which were cultivated in antibiotic free medium.

Plate	*Cells/ well*	*DNA in medium*	*Lipofectamin in medium*	*Plating medium*	*Purpose*
12 wells	50000	1 µg in 200 µl	2 µl in 200 µl	500 µl	Microscopy
6 wells	1 Mio l	4 µg in 200 µl	8 µl in 200 µl	2 ml	Cell lysis

Table 5: Pipetting scheme for chemical transfection with lipofectamin.

2.2.2.7 Cell viability assay

2.2.2.7.1 XTT assay

XTT labeling mixture: XTT labeling reagent and electron coupling reagent (50:1)

The proliferation/viability of cells was measured by a colorimetric assay based on the cleavage of the yellow tetrazolium salt XTT to an orange formazan dye. 10000 cells were seeded out in a 96-well plate and were treated as indicated. Cells were washed with PBS and 100 µl fresh medium as well as 50 µl of the XTT labeling mixture were added to each well in a final XTT concentration of 0.3 mg/ml. The cells were incubated at 37 °C for 4-24 h. The absorbance was measured using the Tecan microplate reader. The formazan dye was measured at a wavelength of 490 nm, the background at a wavelength of 650 nm.

2.2.3 Biochemical methods

2.2.3.1 Protein expression and purification

LB medium: 1 % (w/v) NaCl, 1 % (w/v) peptone, 0.5 % (w/v) yeast extract, pH 7.4; Washing buffer: 300 mM NaCl, 25 mM NaH_2PO_4, 25 mM Na_2HPO_4, pH 8; Equilibration buffer: 30 mM imidazole in washing buffer, pH 8; Elution buffer: 250 mM imidazole in washing buffer, pH 8

Proteins were expressed using a 5 l Fermenter allowing the expression of high amounts of recombinant proteins. 5 l of LB medium, supplemented with selective antibiotics were inoculated at 37 °C with 1 % of an overnight culture of *E. coli* BL21(DE3)pRIL, transformed with the pet15b plasmid. Cells were grown at 37 °C until they had reached the exponential growth phase (OD_{600} of 0.6). The temperature was reduced to 22 °C and 0.5 mM IPTG were added to induce the expression of the protein of interest. Cells were

harvested after 20 h at 4 °C at 5000x g for 10 min. The pellet was washed in 20 ml washing buffer, centrifuged and stored for protein purification at -20 °C. Proteins cloned as His-tagged fusion proteins were purified according to the IMAC (immobilized metal affinity chromatography) technique using the Äkta Prime FPLC system. The pellet was resuspended in 20 ml of equilibration buffer and cells were lysed on ice using a liquid homogenizer applying 10000 psi. The cell suspension was centrifuged at 4 °C at 20000x g for 30 min. The supernatant was loaded on a, with equilibration buffer equilibrated, His-Trap column. The column was washed with washing buffer to avoid unspecific binding. The protein was eluted in 1 ml fractions by adding elution buffer to the column.

2.2.3.2 Generation and purification of antibodies

Antibodies used in this study are listed in Table 1. 40 µg of recombinant protein in 500 µl Complete Freund´s adjuvant (Sigma-Aldrich, Steinheim, Germany) were injected into rabbits, to produce polyclonal antibodies. Every 3-4 weeks injections followed using 40 µg of protein in 500 µl Incomplete Freund´s adjuvant (Sigma-Aldrich). After the third-sixth injection blood was collected. Serum was obtained by incubating the blood for 30-120 min at RT, before centrifuging for 5 min at 5000 rpm. Supernatant was collected and centrifuged for 15 min at 15000 rpm. Serum incubated at 56 °C for 20 min to inactivate the complement immune system. Purification of antibodies was performed using 5 mg of antigen coupled to cyanogenbromid (CnBr) activated sepharose according to the manufacturer. The sepharose was washed with 150 ml PBS, equilibrated with 2 ml coupling buffer and incubated with antigen for 90 min at RT on a rotating table. Free reactive groups were blocked by adding 30 % ethanolamine. After 2 h the column was equilibrated with 6 ml 1 M acidic acid containing 0.5 M NaCl. In a second step 6 ml 0.1 M Tris containing 0.5 M NaCl were applied to the column. In two final equilibration steps 10 ml 10 mM Tris pH 8.8 and 10 ml 10 mM Tris pH 7.5 were administered. 1 ml of the serum was added to the column before washing with 10 ml 10 mM Tris pH 7.5, 10 ml 10 mM Tris pH 7.5 containing 0.5 M NaCl and with 3 ml 10 mM Tris pH 7.5. Antibody was eluted in 500 µl fractions using 5 ml 100 mM glycine pH 2.5. Antibody containing fractions determined by Bradford were rebuffered in PBS using a NAP5 column. After determining the protein concentration the antibody was aliquoted, shock frozen and stored at -20 °C.

2.2.3.3 Biotinylation of purified antibodies

2 mg affinity purified antibody were incubated with 200 µg immunopure NHS-LC-biotin (Pierce) for at least 2 h on ice. The antibody was loaded on a NAP5 column and was eluted with PBS. After determining the protein concentration the antibody was aliquoted, shock frozen and stored at -20 °C.

2.2.3.3.1 Validation of antibodies

All sera and purified antibodies used and produced in this study were validated for specificity by Western Blotting. Antibody stained samples were compared to samples which were stained with antibodies that were prior incubated or rather blocked with 10-200 µg/ ml of the specific antigen. Only antibodies that showed specific staining were used in this study.

2.2.3.4 Protein determination

The total amount of proteins in cell lysates was colorimetrically determined according to Bradford. This assay was performed in a 96-well plate with BSA standards ranging from 0-0.8 mg/ml using the protein-dyeing kit (BioRad, Hercules, USA) according to the manufacturer´s protocol.

The protein concentration of recombinantly expressed and purified proteins was determined spectrophotometrically using the extinction coefficient at 280 nm calculated from the protein sequences using ProtParam (www.expasy.ch).

Protein	***Molecular weight (g/mol)***	***ε coefficient (ε280 1/M*cm)***
hPrx1	22110	18700
hPrx2	21892	21555
hPrx4	30539	36900
hPrx6	25035	22585

Table 6: Molecular weight and extinction coefficients of distinct peroxiredoxins.

2.2.3.5 ELISA

Coatingbuffer: 1.59 g/l Na_2CO_3, 2.93 g/l $NaHCO_3$, 200 mg/l NaN_3, pH 9.6; Blockingbuffer: 1 % BSA in PBS; Washingbuffer: 0.05 % Tween20 in PBS; Incubationbuffer: 0.5 % (w/v) BSA, 0.05 % (v/v) Tween20, 0.02 NaN_3 in PBS; Substratebuffer: 1 M diethanolamin, 0.5 mM $MgCl_2$, 0.02 % NaN_3, pH 9.8

The Enzyme-linked immunosorbent assay (ELISA) is a method to quantify protein expression by the use of specific affinity purified antibodies. A special ELISA microtiter plate was incubated with primary antibody in coatingbuffer over night at 4 °C. The plate was washed 4 times with washingbuffer and was incubated with 200 µl blockingbuffer for 2 h at RT. Standards in the range from 0-32 µg/ml and samples diluted in incubationbuffer were incubated over night at 4 °C. The plate was washed 4 times and after a 2 h incubation with the secondary biotinylated antibody, streptavidin-ALP was added for 1 h at RT. The plate was washed 6 times before adding the substrate p-nitrophenylphosphate in substratebuffer. The colorimetric reaction was measured in a 96-well plate reader (Tecan) at 405 nm.

2.2.3.6 SDS polyacrylamide gel electrophoresis

Loading Dye: 0.3 M Tris/HCl, pH 7, 50 % glycerol, 5 % SDS, 0.1 % bromphenol blue; Runningbuffer: 1 M Tris Base, 1 M HEPES, 0.1 % SDS

Proteins can be separated by size using denaturing SDS polyacrylamid gel electrophoresis (PAGE). 5-30 µg of total protein were diluted and combined with loading dye. Proteins were reduced by a 30 min incubation at RT with 100 mM DTT and 50 mM TCEP if indicated, which was followed by an additional incubation for 10 min at 94 °C. Proteins were separated on a non-reducing SDS-PAGE for 50 min at 120 V using 4-12 % gradient gels (Thermoscientific Fisher, Schwerte, Germany). Gels were washed 3 times with aqua bidest, incubated over night in PageBlue-solution (Fermentas, St. Leon-Rot, Germany) and washed with aqua bidest to remove excessive staining.

2.2.3.7 Western Blot

Transferbuffer: 0.02 % (w/v) SDS, 20 mM Tris, 150 mM glycin, 20 % (v/v) methanol; TBS: 25 mM Tris, 150 mM NaCl, 2.7 mM KCl; TBST: TBS, 0.05 % (v/v) Tween20, pH 7.4; Blockingbuffer: 1 % (w/v) BSA, 5 % (w/v) milk powder in TBST; ECL1 solution: 100 mM Tris, 2.5 mM luminol in DMSO, 0.4 mM p-Coumaracid in DMSO, pH 8.5; ECL2 solution: 100 mM Tris, 0.018 % (v/v) hydrogen peroxide, pH 8.5; Strippingbuffer: 1 g/l SDS, 15 g/l glycerin, 10 ml/l Tween20, pH 2.2; PBS: 8 g/l NaCl, 0.2 g/l KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4

The Western Blot is used to detect specific proteins on a polyacrylamid gel, which have been separated by SDS-PAGE. Proteins were transferred from the gel to a nitrocellulose

membrane (equilibration in aqua bidest) or PVDF membrane (equilibration in methanol) respectively using the wet-blot technique. The transfer was performed on ice for 2 h at 40 V. Membranes were washed 3 times with TBS before blocking for 1 h at RT. Primary antibodies were diluted in blocking buffer as stated in Table 1. Membranes were incubated with the primary antibodies at 4 °C over night on a shaking incubator. Membranes were washed 5 times in TBST before the secondary horseradish-peroxidase(HRP)-coupled antibody was added in blocking buffer without BSA. After a 1 h incubation at RT the membrane was washed 5 times and proteins were detected adding equal amounts of ECL1 and ECL2 to the membranes. The detection was performed using the Chemostar system. Membranes could be stripped from antibodies and analysed for a different protein, by washing twice with strippingbuffer for 10 min, twice with PBS for 10 minutes and twice with TBST for 5 min.

2.2.3.7.1 2-Cys Prx specific redox blot

NEM-lysis buffer: 40 mM HEPES, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1x protease inhibitors, 100 mM N-ethylmaleimide; CHAPS: 2 %; loading dye: 0.3 M Tris-HCl, pH 7, 50 % glycerol, 5 % SDS, 0.1 % bromphenol blue

In order to detect the redox state of 2-Cys Prxs, cells were lysed in NEM-lysis buffer to block free thiols. Samples were diluted and combined with loading dye; no further incubation with any reducing agent was necessary. Proteins were separated by SDS-PAGE (described in 2.2.3.6), immunological labeled with antibodies and analysed using the enhanced chemiluminescence (described in 2.2.3.7).

2.2.3.7.2 Carbonylation Blot

2,4-dinitrophenylhydrazine (DNPH) solution: 10 mM DNPH in 10 % trifluoroacetic acid, stop solution: 2 M Tris, 30 % glycerol and 15 % β-mercaptoethanol

30 µg of total cell extract were incubated with 6 % SDS for 2-3 minutes at 100 °C. DNPH solution was added in a ratio 1:1 for 20 min at 25 °C. One third stop solution was added and mixed gently. Loading dye was added to the samples before following the standard protocol for SDS PAGE (described in 2.2.3.6) and Western blot (described in 2.2.3.7) using a DNP antibody. Samples could be stored at – 20 °C for later analysis.

2.2.3.8 Immunohistochemistry of rat tissues

Anesthetic solution: 28 % (w/v) chloral hydrate, 0.1 ml/100 g of body weight; Perfusion solution/ Fixation solution: 4 % paraformaldehyde in phosphate buffer 0.1 M, pH 7.4; Immersion solution: 5 % sucrose in phosphate buffer 0.1 M, pH 7.4

Male rats were anesthetized and perfused through the abdominal aorta. Brains were dissected and fixated for 2 h, before immersing over night. Tissue sections were cut on an Oxford vibratome. Part of the sections were stained with cresyl violet according to Capani et al. 2009. The other part was incubated with primary antibodies overnight using the dilutions stated in Table 1. After several washing steps with PBS, slides were incubated with a secondary antibody for 2 h at RT, followed by several washingsteps and an additional incubation with a biotin-streptavidin complex (HRP Histo Mark, Caramillo, California, USA) for 1 h at RT. Slides were developed after several washing steps using the AEC substrate kit (Invitrogen Gaithersburg, MD, USA). Images were obtained using a CCD video camera CU-50 (Sony Inc.). Images were analysed using the program Adope Photoshop 8.0 CS3.

2.2.3.9 Immunocytochemistry

Blocking- and Permeabilisation buffer: 10 mM HEPES, 3 % (w/v), 0.3 % (v/v) Triton-X-100 in PBS

Cells were seeded out on glas slides and were incubated at distinct O_2 concentrations. Cells were washed with PBS and mitochondria were stained using 200 nM Mitotracker Deep Red 633. Cells were incubated for 20 min at 37 °C, before washing 3 times with PBS. Cells were fixed with 4 % paraform aldehyde (PFA) for 20 min at RT. Cells were washed 3 times with PBS before incubating with blocking- and permeabilisation buffer for 1 h at RT, before adding antibodies, diluted in blocking- and permeabilisation buffer. After an incubation at 4 °C over night cells were washed three times with PBS and a secondary-Alexa Fluor 488 labeled antibody was added and incubated for 1 h at RT. Cells were washed 3 times with PBS. The actin-cytosceleton was stained using 6.6 nM Phalloidin Alexa Fluor 546 in PBS containing 1 % BSA. After one hour at RT and three washing steps nuclei were stained with 1 ng/ml Dapi. After a 5 min incubation at RT and three washing steps, the cells were mounted using Mowiol. Samples were stored in the dark at 4 °C. Images were obtained using the confocal microscope LCS SP2 (Leica). Deconvolution of images was performed using the program Huygens.

2.2.3.10 Total glutathione assay

TE buffer: 10 mM Tris, 1 mM EDTA, pH 8

The protein content of cell lysates was determined before protein precipitation by incubation with 4 % 5-sulfosalicylic acid over night at 4 °C. Samples were centrifuged for 30 min at 13000 rpm at 4 °C. The supernatant was transferred to a new tube and was neutralised to a pH of 7 by adding 1 M NaOH. Various dilutions in the linear range were analysed for total GSH level by a colorimetric assay based on the reduction of 5,5'-Dithio-bis-(2-Nitrobenzoic acid) (DTNB) to the yellow product TNB, using standards with known GSH concentrations in a range between 0 and 0.4 mM. The reaction mixture contained 1.5 mM NADPH, yeast GR and 1.5 mM DTNB. The reaction was measured as end-point in the Tecan reader at 412 nm.

2.2.3.11 Iron related enzymatic assays

Aconitase buffer: 100 mM Triethanolamin pH 8, 1.5 mM $MgCl_2$, 0.1 % Triton-X-100; Citrate Synthase buffer: 50 mM Tris/HCl pH 8, 100 mM NaCl, 0.5 mM 5,5'-Dithio-bis-(2-Nitrobenzoic acid), 0.1 % Triton-X-100

Cells were fractionated as described before. To determine protein activity, the three fractions were analysed in different colorimetric assays, which were analysed using the Tecan microplate reader. Aconitase-Isocitrate Dehydrogenase activity was measured for 2 h at 340 nm in a 96 well plate. The reaction mix contained aconitase buffer, 1.25 mM NADP, 1 mM cis aconitate and 40 mU/µl isocitrate dehydrogenase. Citrate Synthase activity was measured for 1 h at 412 nm in a 96 well plate. The reaction mix contained citrate synthase buffer, 25 µg acetyl CoA and 25 µg oxalacetate (Stehling et al. 2007).

2.2.3.11.1 Measuring ferrochelatase activity

Cells were harvested, washed twice in PBS and were frozen as cell pellets in liquid nitrogen. Cells were thawn and resuspended in 20 mM HEPES, pH 7.4, 50 mM KCl, 1 mM $MgSO_4$, 0.6 M sorbitol. 200 µg of protein, 1 mM ascorbate, 2 mM succinate, 2.5 µM deuteroporphyrine and 1 µCi/10 µl ^{55}Fe-Chloride were incubated for 15 min at 30 °C, gently shaking. Samples were put on ice and stop-solution (100 mM $FeCl_2$ in 5 M HCl) and butylacetate were added. Samples were vortexed twice for 30 sec and were centrifuged for 5 min at 14000 rpm, before analysing using a scintillation counter.

3. Results

3.1 Generation of new tools

In order to detect and quantify protein levels in samples of distinct origins, specific polyclonal antibodies were generated (3.1.1) and validated for Western blot analysis (described in 2.2.3.7). The specificity of the antibodies against antigens derived from human, mouse and rat was determined. In general, the Western blot is considered a strong tool to detect the protein of interest in samples containing a mixture of antigens, such as cell or tissue extracts. However, this method has limitations when it comes to proteins with low concentrations, such as Grx2. Therefore, a Grx2-specific sandwich ELISA (described in 2.2.3.5) was established (3.1.2), which constitutes a more sensitive method, also based on antigen detection by a specific antibody.

Plasmids for protein overexpression in mammalian cell lines were generated (3.1.3), using the pExpress plasmid (Figure 10). In addition, the RNA interference technique was established in the work group, to specifically knock-down the protein expression of various oxidoreductases (3.1.3). Table 7 summarizes the new established tools.

3.1.1 Generation and evaluation of new antibodies

Human Prxs 1, 2, 4 and 6 were cloned from human cDNA (Biocat, Heidelberg, Germany) using the primer pairs listed in Table 2. The PCR products were integrated into the pGEM-T plasmid (Figure 8), before subcloning them into the pet15b plasmid (Figure 9) for protein expression. After verification by sequencing, plasmids were transformed into the BL21(DE3)pRIL strain of *E. coli* (described in 2.1.7.1). Bacteria were cultivated and proteins were expressed as His-tag proteins using a Fermenter (described 2.2.3.1) and purified using the Äkta Prime FPLC system (2.2.3.1). Rabbits were immunized by several injections of 40 µg protein (described in 2.2.3.2). Blood samples were taken and the serum was isolated. After heat inactivation, the serum was evaluated by Western blot using 20 µg of human, mouse and rat cell extracts and tissues, which were prior to analysis reduced by 100 mM DTT. All generated antibodies led to clear and specific bands at the estimated protein sizes. Using antibodies preincubated with 100 µg of the respective antigen, resulted in a complete loss of signal (data not shown). Figure 14 exemplary shows the results for the 22 kDa hPrx1.

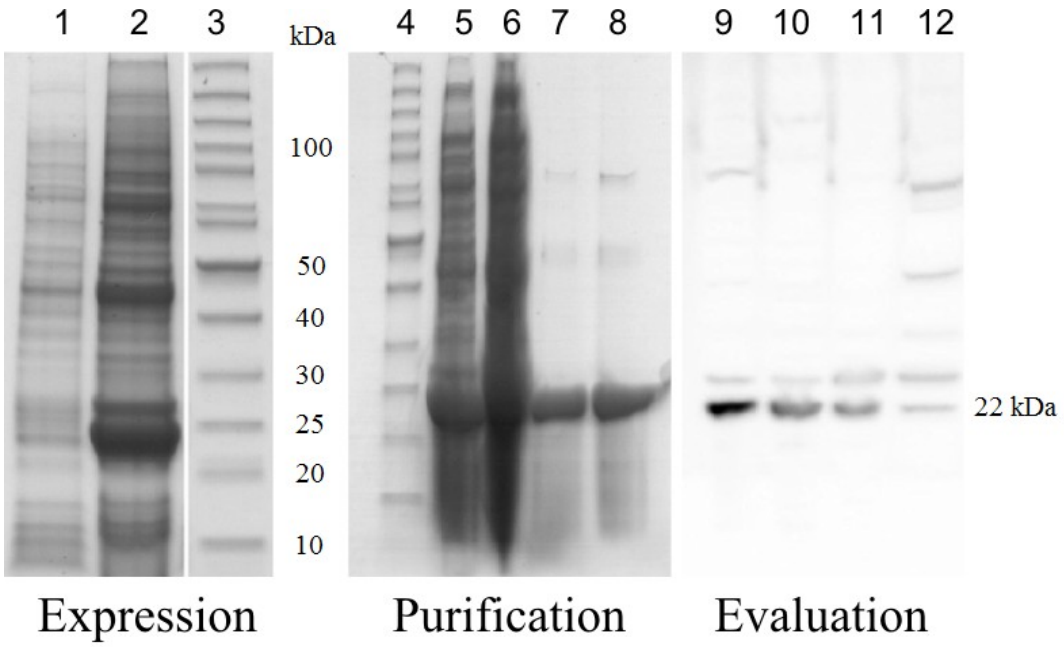

Figure 14: Expression and purification of the 22 kDa hPrx1, showing coomassie-stained SDS gels, as well as antibody validation by Western blot. Protein expression (1-3) and purification (4-8) of BL21(DE3)pRIL *E. coli* cells, transfected with hPrx1-pet15b. Purified protein was injected into rabbits producing polyclonal anti-hPrx1 serum, tested for specificity by Western blot using distinct antigens (9-12). 1: Crude cell extract prior IPTG induced protein expression; 2: Crude cell extract after IPTG induced protein expression; 3+4: protein marker; 5: crude cell extract; 6: flow through; 7: elution of the main fraction using 200 mM imidazole; 8: elution of the side fraction; 9: human HeLa cell extract; 10: murine MIN6 cell extract; 11: rat INS1 cell extract; 12: murine brain extract.

3.1.2 Grx2-specific sandwich ELISA

A Grx2-specific sandwich ELISA was established, to quantify protein levels in samples of distinct origins such as cell lysates, tissues or human patient samples. Mouse Grx2c antiserum was affinity-purified and biotinylated (described in 2.2.3.3). Various concentrations of primary and secondary antibodies ranging from 0.5 µg/ml to 10 µg/ml, as well as antigen standards of recombinantly expressed human, mouse or rat protein in the range of 0-100 µg/ml were tested (data not shown). Subsequently, all experiments were performed using 0.5 µg/ml primary, 0.5 µg/ml secondary antibody and protein standards in the range of 0-32 µg/ml of human, mouse or rat Grx2. Even though the antibody used in this study was directed against the mouse protein, it crossreacted with human and rat antigens with essentially the same specificity and a sensitivity of below 5 ng/mg total protein. Since the antibody was directed against the core part of the protein it was not possible to distinguish between the different isoforms of Grx2, but rather measure total levels of the oxidoreductase.

Figure 15 shows a standard curve for human Grx2 and illustrates Grx2 levels in HeLa cells (2.1.7.2) transfected with siRNA against all isoforms of Grx2 and a Grx2c-pExpress plasmid, compared to wildtype cells.

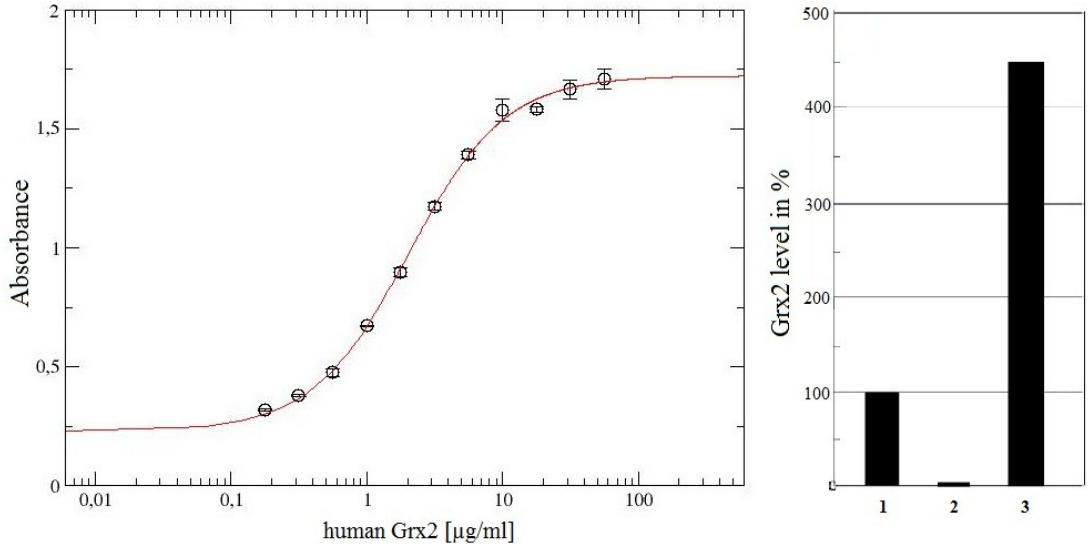

Figure 15: Grx2-specific sandwich ELISA. Left: standard curve using human protein standards in the range of 0-32 µg/ml. Right: Grx2 levels in HeLa WT cells (1), HeLa cells transfected with Grx2-specific siRNA (2) and a Grx2c-pExpress plasmid (3).

3.1.3 Overexpressing and silencing intracellular protein levels

Human peroxiredoxins 1-6 were cloned from human cDNA (Biocat, Heidelberg, Germany) using the primer pairs listed in Table 2. The PCR products were amplified using the pGEM-T plasmid and subsequently transferred into the pExpress plasmid. After verifying the plasmids by sequencing (Seqlab, Göttingen, Germany), 3.5 million HeLa cells were transfected with 15 µg of DNA using the electroporation technique (described 2.2.2.6.1). HeLa cells transfected with an empty pExpress plasmid were used as control. Overexpression was analysed after 24 h by the Western blot technique. 20 µg of total cell extracts were reduced by a 20 min incubation with 100 mM DTT at RT, which was followed by a 10 min incubation at 94 °C. Proteins were separated by denaturing SDS-PAGE (described in 2.2.3.6), blotted on nitrocellulose membranes and analysed for protein levels using specific primary antibodies (stated in Table 1), HRP-labeled secondary antibodies (stated in Table 1) and the chemiluminescence method. Overexpression in HeLa cells was detected for all Prxs, which was at least two-fold the level of protein, compared to the control (data not shown).

In addition, we have performed site-directed mutagenesis (described in 2.2.1.2.1) to integrate mutations into the hGrx2c gene incorporated into the plasmid pEGFP-N1 (Figure 11). Human Grx2 was identified as an iron-sulfur protein. The [2Fe2S] cluster was shown to be coordinated by the N-terminal active site cysteinyl residues from two Grx2 monomers and two molecules of reduced GSH, which were non-covalently bound to hGrx2. Mutating the N-terminal Cys74 to a serine rendered the protein unable to bind the metal cofactor. Introducing the Thr95Arg mutation into the Grx2 gene attenuated the binding of GSH towards Grx2, affecting the stability of the cluster (Lillig et al. 2005), (Berndt et al. 2007).

Within this study, the use of siRNAs against hGrx1, hGrx2, hTrx1 and hTrx2, as well as a scrambled control siRNA was established. HeLa cells were transfected with 15 µg of three different test siRNAs per gene/protein using the electroporation method. Protein knock-down was analysed in total cell extracts after three days, as well as after another three days following a second transfection. The protein content was measured photometrically according to Bradford (described in 2.2.3.4). 20 µg of total cell extracts were analysed for protein amounts by Western blot, as described above. In the case of hGrx2, the protein level was quantified by the Grx2-specific sandwich ELISA which was established and optimized as described before (3.1.2). Figure 16 exemplary shows the protein expression of HeLa cells transfected with test siRNAs against hTrx2 (Trx2si A,B,C). Trx2si B and C led to a significant reduction of Trx2 protein levels, whereas Trx2si A rather led to a slight increase. The first transfection already resulted in an efficient reduction, which was even further decreased by the second transfection. HeLa cells treated with scrambled siRNA, as well as wildtype HeLa cells were used as control. Table 4 shows the sequences of the siRNAs which led to the lowest protein amount, including Trx2si C, and were used throughout this study.

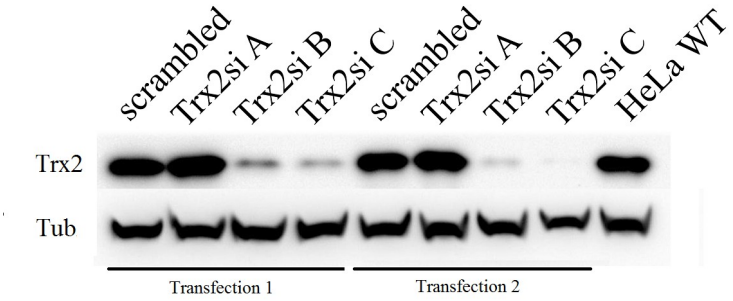

Figure 16: HeLa cells were transfected with three test siRNAs against hTrx2 (Trx2si A, Trx2si B, Trx2si C). After 3 days cells were transfected a second time. Scrambled siRNA treated cells as well as wildtype HeLa cells were used as transfection control. Tubulin was used as loading control.

Tool	***Purpose***	***Chapter***
Antibody mGrx2c, affinity purified	Protein detection by ELISA	3.1.2
Antibody mGrx2c, biotinylated	Protein detection by ELISA	3.1.2
Antiserum hPrx1	Protein detection by Western blot and IHC	3.1.1
Antiserum hPrx2	Protein detection by Western blot and IHC	3.1.1
Antiserum hPrx4	Protein detection by Western blot and IHC	3.1.1
Antiserum hPrx6	Protein detection by Western blot and IHC	3.1.1
Grx2-specific ELISA	Protein detection by ELISA	3.1.2
pet15b hPrx1 plasmid	Protein expression in *E. coli*	3.1.1
pet15b hPrx2 plasmid	Protein expression in *E. coli*	3.1.1
pet15b hPrx4 plasmid	Protein expression in *E. coli*	3.1.1
pet15b hPrx6 plasmid	Protein expression in *E. coli*	3.1.1
pExpress hPrx1 plasmid	Protein overexpression in cells	3.1.3
pExpress hPrx2 plasmid	Protein overexpression in cells	3.1.3
pExpress hPrx3 plasmid	Protein overexpression in cells	3.1.3
pExpress hPrx4 plasmid	Protein overexpression in cells	3.1.3
pExpress hPrx5 plasmid	Protein overexpression in cells	3.1.3
pExpress hPrx6 plasmid	Protein overexpression in cells	3.1.3
pGFP-N1 hGrx2 S74P	Protein overexpression in cells	3.1.3
pGFP-N1 hGrx2 T95R	Protein overexpression in cells	3.1.3
Protein hGrx2c	Protein standards for ELISA	3.1.2
Protein hPrx1	Antiserum production in rabbits	3.1.1
Protein hPrx2	Antiserum production in rabbits	3.1.1
Protein hPrx4	Antiserum production in rabbits	3.1.1
Protein hPrx6	Antiserum production in rabbits	3.1.1
Protein mGrx2c	Protein standards for ELISA	3.1.2
Protein rGrx2c	Protein standards for ELISA	3.1.2
siRNA hGrx1	Silencing protein expression	3.1.3
siRNA hGrx2	Silencing protein expression	3.1.3
siRNA hTrx1	Silencing protein expression	3.1.3
siRNA hTrx2	Silencing protein expression	3.1.3
siRNA scrambled control	Silencing protein expression	3.1.3

Table 7: Tools generated within this study, listed in alphabetic order.

3.2 Does mitochondrial hGrx2 reduce Prx3 in vivo?

Prior to this study, *in vitro* kinetics of recombinantly expressed human proteins revealed that the mitochondrial Grx2 could donate electrons to the 2-Cys Prx3, but not to the atypical 2-Cys Prx5. So far, the reduction of mammalian peroxiredoxins was only ascribed to the Trx system. To analyse if Prx3 is a substrate for Grx2 *in vivo*, HeLa cells were transfected twice with specific siRNAs against hGrx2 and hTrx2, reducing the protein levels to 5-20 % compared to control cells treated with scrambled siRNA, analysed by ELISA or Western blot (Figure 17C), respectively.

Prior to cell lysis, cells were treated with 100 mM N-ethylmalemeide (NEM), an alkylating agent which exclusively and irreversibly binds to reduced thiol groups, preventing them from further oxidation. Cell extracts were analysed via a specific redox blot for 2-Cys Prxs (described in 2.2.3.7.1). Due to an intermolecular disulfide, oxidised Prx3 could be identified as a dimeric band at 46 kDa and reduced, monomeric Prx3 at 23 kDa. 10 µg of total cell extracts were separated via denaturing SDS-PAGE, blotted on PVDF membranes and analysed using a specific, polyclonal primary antibody against Prx3, HRP-labeled secondary antibody and the chemiluminescence method. Silencing the expression of only Grx2 or Trx2 did not significantly increase the levels of oxidised Prx3, compared to scrambled siRNA control cells. However, transfecting HeLa cells with both siRNAs simultaneously led to the accumulation of oxidised Prx3 (Figure 17A,B). To exclude that the oxidation of Prx3 was a secondary effect due to generally increasing oxidative conditions resulting from the absence of the two mitochondrial redoxins and major antioxidants, cell extracts were analysed for oxidative modifications of proteins, i.e. glutathionylation and carbonylation.

Glutathionylation constitutes the reversible, covalent binding of GSH to cysteinyl residues of target proteins. It is regarded as cellular response to changes in the redox state, transducing oxidative stimuli into adjusted protein function. In addition, it was proposed that glutathionylation could be a mechanism to protect proteins from irreversible oxidation (Fratelli et al. 2004). Glutathionylation was analysed by the Western blot technique using a specific antibody against GSH.

Carbonylation on the other hand is an irreversible modification, which generally is used as marker for protein modifications induced by increased oxidative conditions.

In order to measure carbonylation (described in 2.2.3.7.2), 30 µg of cell extracts were incubated with 2,4-dinitrophenylhydrazine, forming protein-bound 2,4-dinitrophenylhydrazones (DNP). These hydranozones were analysed by Western blot using a specific DNP antibody. Neither the detection of glutathionylated proteins, nor the analysis of protein carbonylation showed any differences in the amount of oxidative modified proteins in the differently treated cell extracts (data not shown).

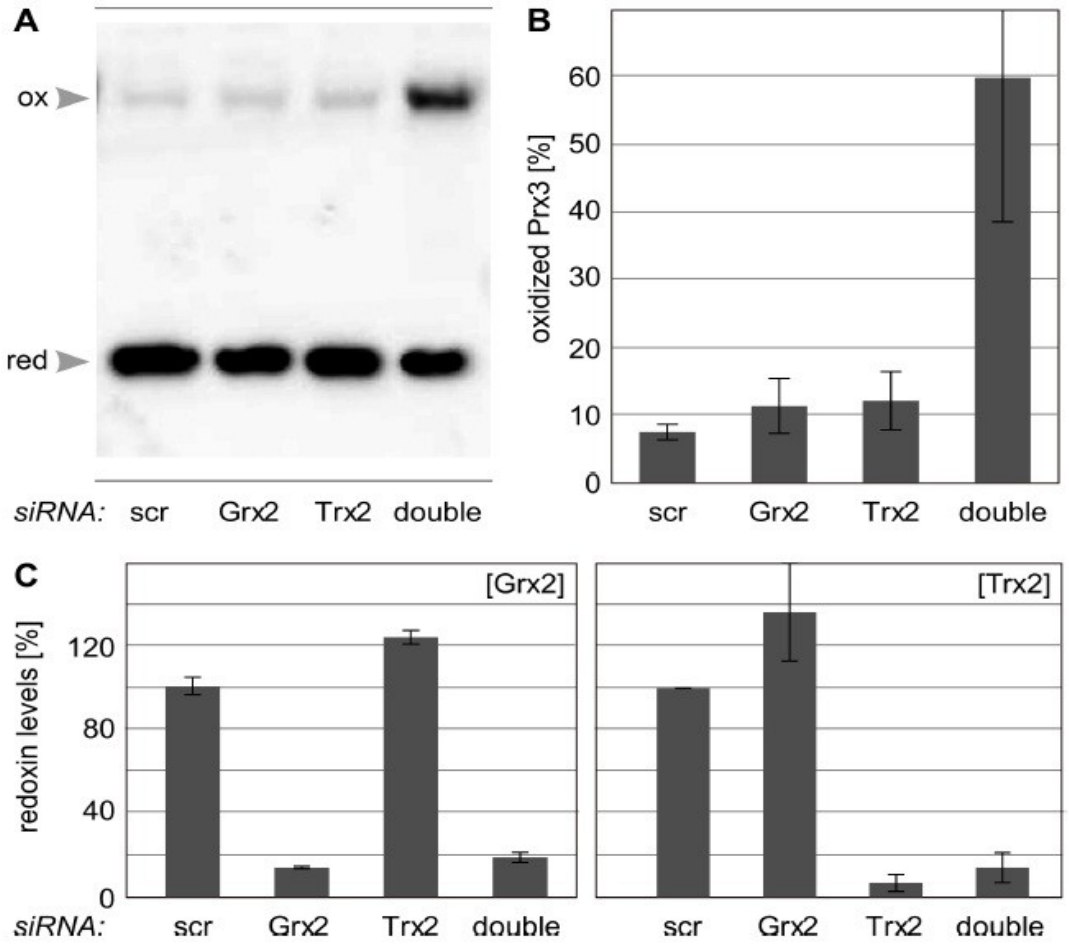

Figure 17: Prx3 is a substrate for the mitochondrial Trx and Grx systems. A) The redox state of hPrx3 was analysed in HeLa cells with decreased expression levels of Grx2, Trx2 or both oxidoreductases, due to transfection with specific siRNAs. HeLa cells transfected with unspecific scrambled (scr) siRNA were used as control. Cell extracts were, prior to cell lysis, treated with NEM to block free thiol groups and analysed by Western blot. Due to an intermolecular disulfide, oxidised Prx3 could be detected as a dimeric band at 46 kDa and reduced Prx3 as monomeric band at 23 kDa. B) The results from three independent Western blots were quantified using ImageJ. Oxidised Prx3 is constituted in %. C) Grx2 levels were analysed by a specific sandwich ELISA, and Trx2 levels by Western blot. The diagrams represent data from three independent experiments (Hanschmann et al. 2010).

Redox reactions are rapid and specific signaling events. Reducing or oxidising compounds respectively, can lead to posttranslational modifications of cysteinyl residues. The general kinetics behind these events are missing, i.e. how fast the oxidation of a certain protein upon an oxidizing stimuli is, as well as the subsequent reduction or regeneration.

To answer these questions for the oxidation and subsequent reduction of hPrx3 *in vivo*, HeLa cells were treated with 50 µM H_2O_2 – a concentration which led to a rapid, but fully reversible oxidation of the peroxidase. HeLa cells were harvested, washed once with PBS and were incubated with H_2O_2, diluted in PBS buffer. After different incubation times the cells were centrifuged at 2000 rpm for one minute, incubated in NEM-lysis buffer for 15 minutes and shock frozen in liquid nitrogen. Cell extracts were analysed for total protein amount according to Bradford and for the redox state of hPrx3 via the above described 2-Cys Prx-specific redox blot.

Figure 18 illustrates the quantification of the Western blot bands using the programs ImageJ and Grace. Without the oxidizing stimuli, HeLa cells contained 87 % reduced hPrx3. Upon H_2O_2 treatment the peroxidase was rapidly oxidised. After an incubation time of two minutes, less than 30 % of the peroxidase were in the catalytically active state. After five minutes the oxidised protein recovered to 35 %, after another five minutes to 50 %. 30 minutes after the H_2O_2 treatment the peroxidase recovered, with more than 80 % of the protein being in the monomeric, catalytically active form.

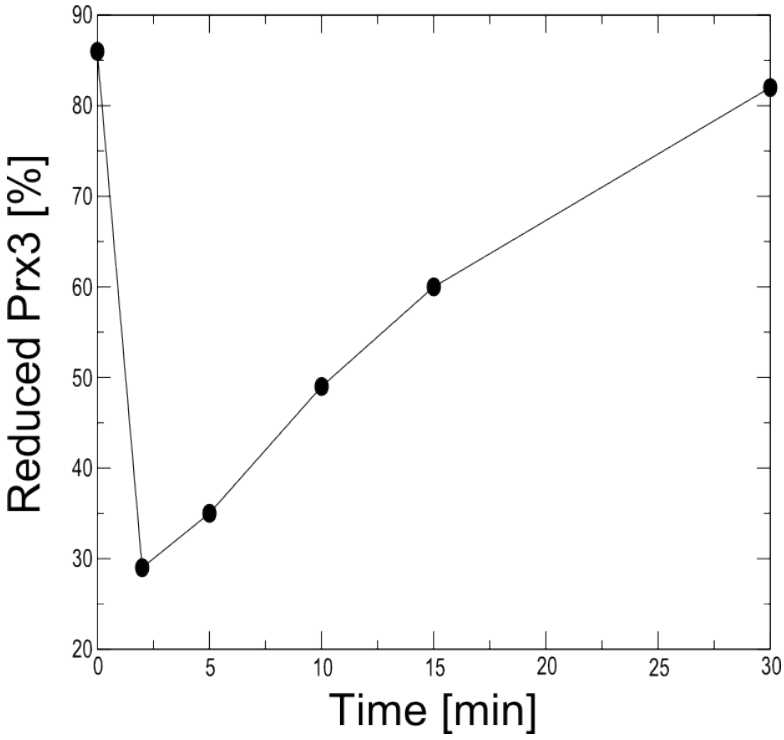

Figure 18: Oxidation of intracellular hPrx3 by H_2O_2 in HeLa cells. HeLa cells were treated with 50 µM H_2O_2. The oxidation was stopped at different time points by adding the alkylating agent N-ethylmalemeide, which prevented oxidation of free thiol groups irreversibly. Cell extracts were analysed by Western blot. Densitometric analysis was performed using ImageJ and Grace.

3.3 Trx proteins in iron homeostasis

Iron is an essential trace element functioning for instance in the mitochondrial respiratory chain, the tricarboxylic acid cycle and DNA synthesis as cofactor of proteins such as hemoglobin, cytochromes and iron-sulfur proteins. Iron regulation needs to be tightly controlled because iron starvation and also iron overload constitute toxic conditions for cells.

Glutaredoxins were shown to be involved in iron regulation and FeS cluster assembly in various organisms. However, the only report in mammals described a homozygous silent mutation in the Grx5 gene of a human patient, suffering from sideroblastic-like microcytic anemia and iron overload (Camaschella et al. 2007).

Here, the impact of silencing the expression of distinct Trx family proteins on the cellular iron regulation and the biosynthesis of FeS clusters was examined. HeLa cells were transfected twice with specific siRNAs, reducing the protein levels of cytosolic/nuclear dithiol Trx1, mitochondrial dithiol Grx2 and cytosolic/nuclear monothiol Grx3 to less than 5-10 % compared to control cells treated with scrambled siRNA. Crude cell extracts were analysed for protein levels of the iron regulatory protein IRP1 and the target proteins ferritin and the transferrin receptor using the Western blot technique. IRP1 contains a [4Fe4S] cluster and catalyses the conversion from citrate to isocitrate in the tricarboxylic acid cycle. Upon iron deficiency the cofactor is lost and the protein binds to IREs on the mRNA of distinct targets, upregulating

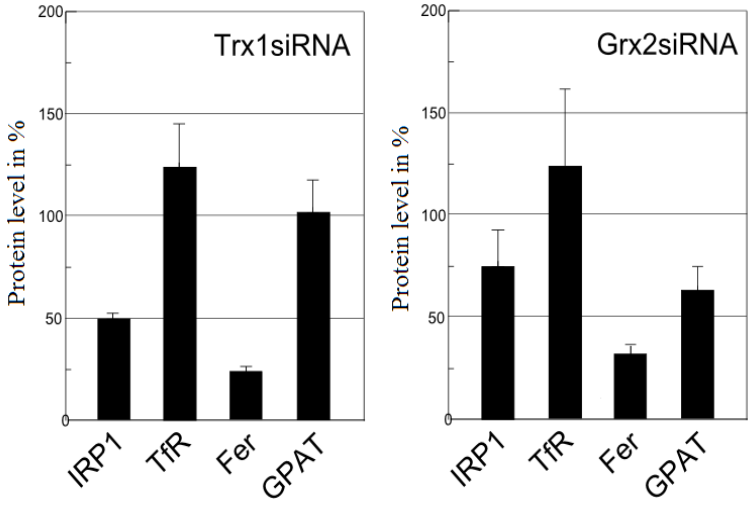

Figure 19: Iron regulation and FeS cluster synthesis in HeLa cells with reduced Trx1 and Grx2 protein levels. Cells transfected with scrambled siRNA were used as control. Protein expression levels of tubulin, the cytosolic iron-regulatory protein 1 (IRP1), transferrin receptor (TfR) and ferritin (Fer), as well as the cytosolic FeS protein glutamine phosphoribosylpyrophosphate amidotransferase (GPAT) were detected by Western Blot and were quantified. Data are presented in %, regarding scrambled control cells as 100 %.

for instance the expression of the TfR and repressing the translation of ferritin. Moreover, the cytosolic glutamine phosphoribosylpyrophosphate amidotransferase (GPAT), coordinating a [4Fe4S] cluster was analysed. The enzyme catalyses the first reaction of the *de novo* purine pathway. GPAT is regarded as a marker protein for the intact biosynthesis of iron-sulfur cluster coordinating proteins, because it is less stable and quickly degraded without the iron cofactor (Zhou et al. 1992), (Stehling et al. 2008).

20 µg of total cell extracts were separated by SDS-PAGE without prior protein reduction. Proteins were transferred on nitrocellulose membranes and detected by specific primary antibodies, HRP-coupled secondary antibodies and the chemiluminescence method. Antibodies and dilutions are listed in Table 1.

Western blot analysis of Trx1 depleted cells revealed an affected iron regulation, but no effect on FeS cluster biosynthesis (Figure 19). Compared to control cells, IRP1 levels were reduced to 50 % and ferritin decreased to approximately 25 %. The amount of the TfR was increased to 124 %. Knock-down of Trx1 showed no changes in GPAT levels (Figure 19).

Iron regulation in Grx2 depleted cells was not comprehensibly affected. IRP1 levels were slightly decreased, the TfR was slightly increased and ferritin was reduced to 32 %, compared to scrambled control cells. However, GPAT was downregulated to 60 %, indicating that Grx2 knock-down affected the biosynthesis of FeS cluster-coordinating proteins (Figure 19).

Knock-down of Grx3 in HeLa cells had the strongest effect on cellular iron homeostasis, impairing not only general iron regulation, but also FeS cluster biosynthesis. IRP1 was downregulated to less than 50 %, ferritin to 12 % and the TfR was upregulated to more than 200 %. The expression level of GPAT was reduced to 50 % (Figure 20A,B).

Because Grx3 depletion resulted in the strongest phenotype, we focussed on analysing the effects of reduced protein levels of the monothiol, multidomain glutaredoxin. Since the protein amount of IRP1 was decreased to 50 %, the activity of the regulatory protein was analysed in Grx3 depleted cells, which were fractionated using digitonin (described in 2.2.2.5). Protein activity was measured in total cell lysates, cytosolic fractions and pellet fractions, containing membrane enclosed compartments including nuclei and mitochondria. Enzyme activity of the cytosolic aconitase was measured in an enzymatic assay following NADP consumption at 412 nm in a 96-well plate reader

(Tecan) (described in 2.2.3.11). Activity of citrate synthase, another enzyme of the tricarboxylic acid cycle which does not coordinate an iron-sulfur cluster and was not affected by Grx3 depletion was used as a control (data not shown).

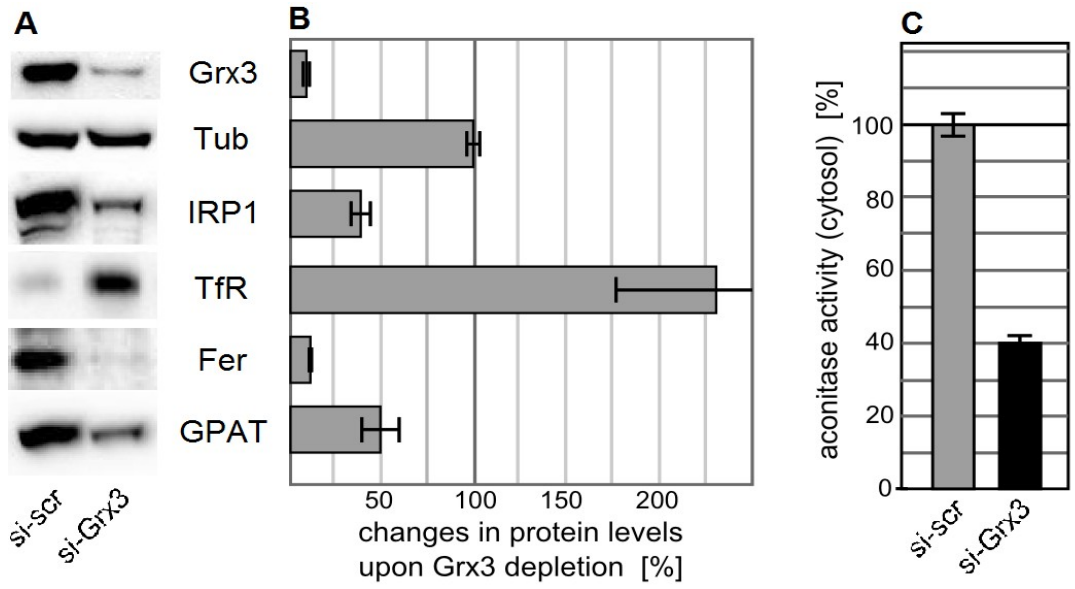

Figure 20: Knock-down of hGrx3 affects cytosolic iron homeostasis and biosynthesis of FeS clusters. HeLa cells were transfected with siRNA against hGrx3 and scrambled siRNA as control. Protein levels of Grx3, tubulin, iron-regulatory protein 1 (IRP1), transferrin receptor (TfR) and ferritin (Fer), as well as the FeS protein GPAT were detected by Western blot (A) and were quantified using ImageJ (B). Cells were fractionated and cytosolic aconitase activity (C) was measured. Enzyme activity is stated in %, regarding scrambled control cells as 100 % (modified from Haunhorst et al. 2011, unpublished manuscript, compare to 4.3).

Down-regulation of Grx3 resulted in diminished protein activity of cytosolic aconitase of 40 % compared to control cells (Figure 20C). In order to determine if Grx3 depletion also affected mitochondrial proteins, the activity of mitochondrial FeS and heme proteins was investigated. Activity of the mitochondrial heme protein cytochrome *c* oxidase, i.e. complex V of the respiratory chain, was measured photometrically in the pellet fraction. Knock-down of Grx3 led to a decrease in enzyme activity of approximately 20 % (Figure 21A). A similar reduction in enzyme activity was determined for the mitochondrial ferrochelatase, which coordinates a FeS cluster that is essential for protein activity. It catalyses the last step in the biosynthesis of heme. Protein activity was determined in whole cells, following the insertion of ^{55}Fe in deuteroporphyrine using a scintillation counter. Grx3 depleted HeLa cells showed a reduced enzyme activity of 80 %, compared to control cells (Figure 21B). Mitochondrial aconitase activity was essentially the same, compared to control cells

(Figure 21C). However, when these data were correlated to the actual protein levels of mitochondrial aconitase (Figure 21D), determined by Western blot and quantified by ImageJ, a decrease in specific activity to approximately 80 % was determined (data not shown).

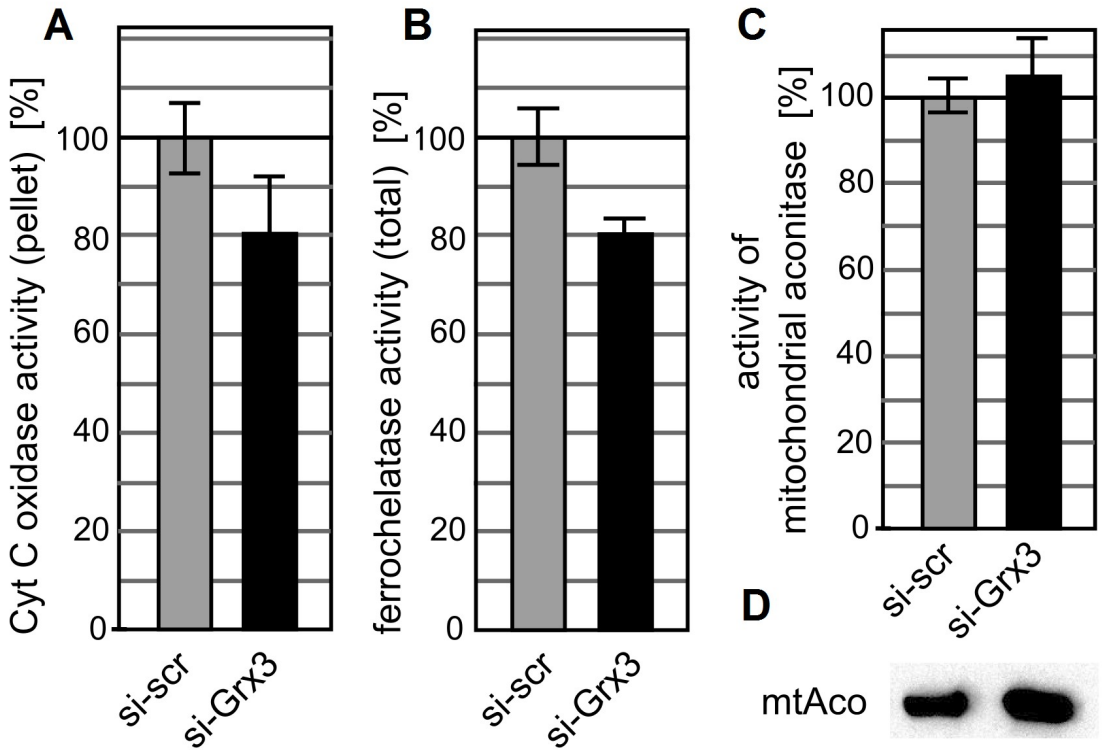

Figure 21: Knock-down of Grx3 affects mitochondrial iron homeostasis and biosynthesis of FeS and heme proteins. HeLa cells were transfected with siRNA against Grx3 and scrambled siRNA as control and fractionated using digitonin. Activity of the heme protein cytochrome *c* oxidase (A), ferrochelatase, which depends on the insertion of a FeS cluster (B) and mitochondrial aconitase (C) were measured. Western blot of control and Grx3-depleted cells shows the protein levels of mitochondrial aconitase (mtAco) (D) (Haunhorst et al. 2011, manuscript, compare to 4.3).

3.4 Localization of Trx family proteins in the normal and the asphyxic brain

In a cooperative project with the group of Prof. MD, PhD Francisco Capani from the University of Buenos Aires, the distribution of thioredoxin family proteins (Trx1, Trx2, TrxR1, TrxR2, Grx1, Grx2, Grx3, Grx5, Prx1, Prx2, Prx3, Prx4, Prx5, Prx6) and related proteins (Txnip, γGCS) was studied in the rat central nervous system by immunohistochemistry (described in 2.2.3.8; performed by Laura Aon-Bertolino). The cerebellum, striatum, hippocampus, spinal cord, substantia nigra, cortex and retina – regions of the brain which are most sensitive to an ischemic insult – were analysed. The aim of this project was to examine the localisation of the above mentioned proteins in order to understand why various cell types differ in their susceptibility towards an ischemia/reperfusion insult. All antibodies used within this study are stated in Table 1 and were validated for rat tissue. 30 µg of cell extracts of the rat insulinoma cell line INS1 were reduced with 100 mM DTT for 20 min at RT and another 10 min at 94 °C. In addition, 50 mM TCEP were added to ensure better disulfide reduction. Samples were analysed for protein expression by Western blot. The antibody against γGCS recognized the catalytic subunit of the protein.

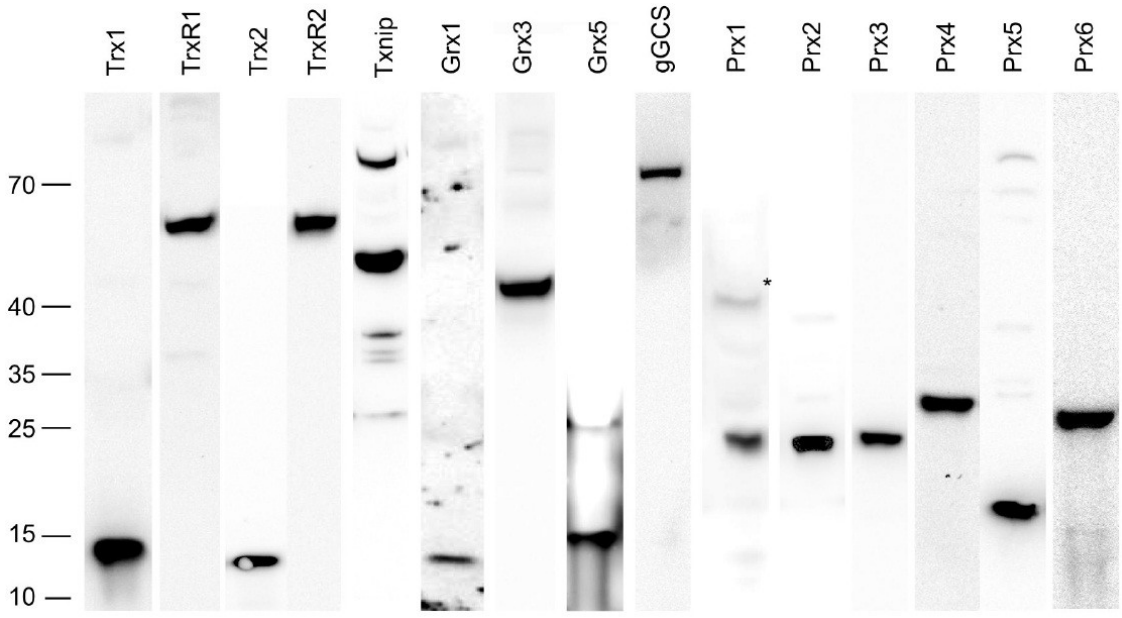

Figure 22: Antibody specificity in rat cell extracts. 30 µg of cell extracts of rat insulinoma cells (INS1) were reduced with 100 mM DTT and 50 mM TCEP, separated by denaturing SDS-PAGE, blotted on nitrocellulose membranes, stained using primary antibodies, HRP-coupled secondary antibodies and the enhanced chemiluminescence method. The scale to the left indicates the protein size in kDa (Aon-Bertolino et al. 2011).

All antibodies detected the rat antigen and showed specific bands at the expected sizes (Figure 22). Weak bands e.g. in the case of Prx1 indicated insufficient protein reduction. All proteins were detected throughout the brain and showed a tissue and cell type-specific staining. Figure 32 exemplary shows the distribution of redoxins in the hippocampus.

We have furthermore collected samples from a rat model mimicking perinatal asphyxia (described in 2.1.8.1), a condition where a newborn suffers from oxygen deprivation. Trx family proteins have been implicated in various diseases, including conditions related to oxygen deprivation. To understand the role of the protein family in hypoxia, we have analysed the expression and distribution of these proteins.

Fullterm pregnant rats were rendered unconscious by CO_2 inhalation and were decapitated. The uterus horns were isolated and incubated in a waterbath at 37 °C. After 19 minutes the uterus horns were opened, the pubs were cleaned and monitored for one hour while stimulating breathing by performing tactile intermittent. One hour to seven days after the procedure, male animals were killed by CO_2 inhalation, the brain was extracted and the hippocampus was isolated. Part of the tissue was homogenized and prepared for analysis by Western blot and ELISA. The other part was cut into sections for IHC analysis, performed by Laura Aon-Bertolino. Expression and distribution of Grx1, Grx2, Trx1 and Trx2 were determined. Tissues of untreated pubs were used as control.

Grx2, Trx1 and Trx2 showed the strongest staining four hours after the induction of hypoxia, compared to control samples. In the samples, collected after seven days, no significant changes in protein expression were detected (data not shown).

3.5 Trx family proteins in ischemia/ reperfusion injury

HEK293 cells (2.1.7.2) - human embryonic kidney cells – were used as a cell model to analyse if cellular protein levels and localisation of members of the Trx protein family depend on the environmental oxygen concentration. Cells were cultivated in an atmosphere containing distinct concentrations of oxygen for 24 h: 20 % O_2, a concentration commonly used for cell culture, 1 % and 0.1 %. Cells were washed with PBS buffer, harvested and lysed in NP40-lysis buffer. Protein levels of Trx family proteins were analysed by the Western blot technique using 10-20 µg of cell extracts, reduced with 100 mM DTT and 50 mM TCEP as described above. Various proteins, commonly used as loading controls, i.e the cytosceletal components tubulin and actin, as well as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the enzyme catalysing the sixth step of glycolysis, were also analysed. All proteins responded to the hypoxic insult. GAPDH for instance, was upregulated with decreasing O_2 concentrations (Figure 23). However, the overall protein concentration of all samples was thoroughly analysed by Bradford. Figure 23 summarizes the results of the Western blot analysis. Generally, Trx family proteins showed a complex response to the hypoxic insults. Protein levels of Grx1, Grx5, Prx2, TrxR1 and Nrx increased,

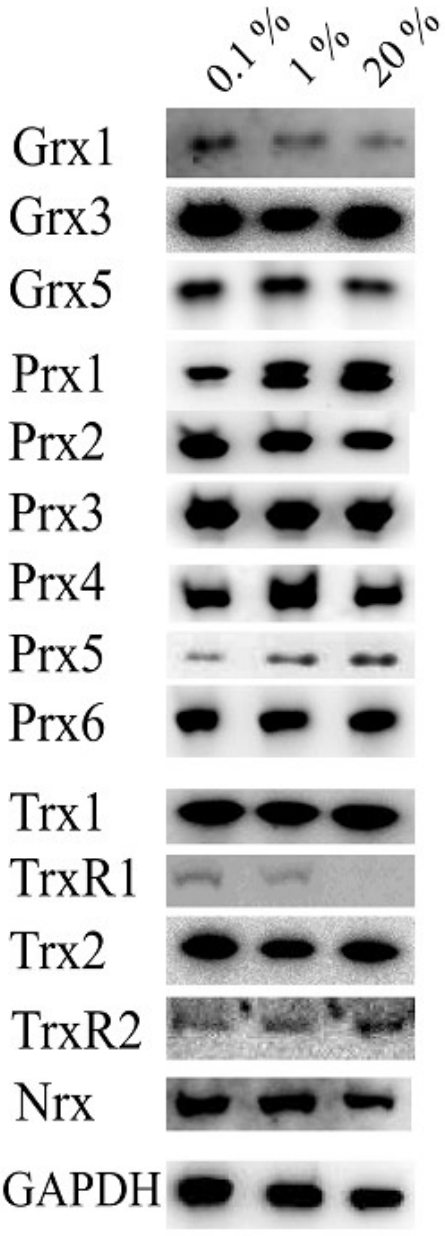

Figure 23: Protein expression of Trx family proteins is sensitive to oxygen concentrations. HEK293 cells were grown in an atmosphere containing distinct O_2 (0.1 %, 1 %, 20 %) concentrations. Protein levels were analysed by Western blot, using 20 µg of total cell extracts, preincubated with 100 mM DTT and 50 mM TCEP.

whereas the levels of Prx1, Prx5 and TrxR2 decreased. Grx3, Trx1 and Trx2 showed similar levels at 0.1 % and 20 %, but were downregulated at 1 %, whereas Prx4 was upregulated at 1 %. Prx3 and Prx6 did not display any significant changes. We subsequently analysed Trx family proteins in a mouse model, where renal ischemia/reperfusion injury (2.1.8.2) was induced by clamping the pedicle of the right kidney for 30 min and releasing it afterwards for another 24 h. This animal model was established in the AG Lillig by Dr. José Godoy, who performed all animal experiments. Blood and urine specimens, as well as the ischemic and the contralateral kidney were used for analysis. Sham operated mice, animals where the kidney was exposed for 30 min, but was not clamped, were used as control. Numerous parameters including consistent body weight, decreased urea elimination, proteinuria, increased segmented nuclei cells, indicating the start of an inflammation as well as histological changes of the kidney, such as loss of brush borders, tubular cell flattening and luminal obstruction indicated the success of the procedure (Godoy et al. 2011b, Table 9, Figure 33).

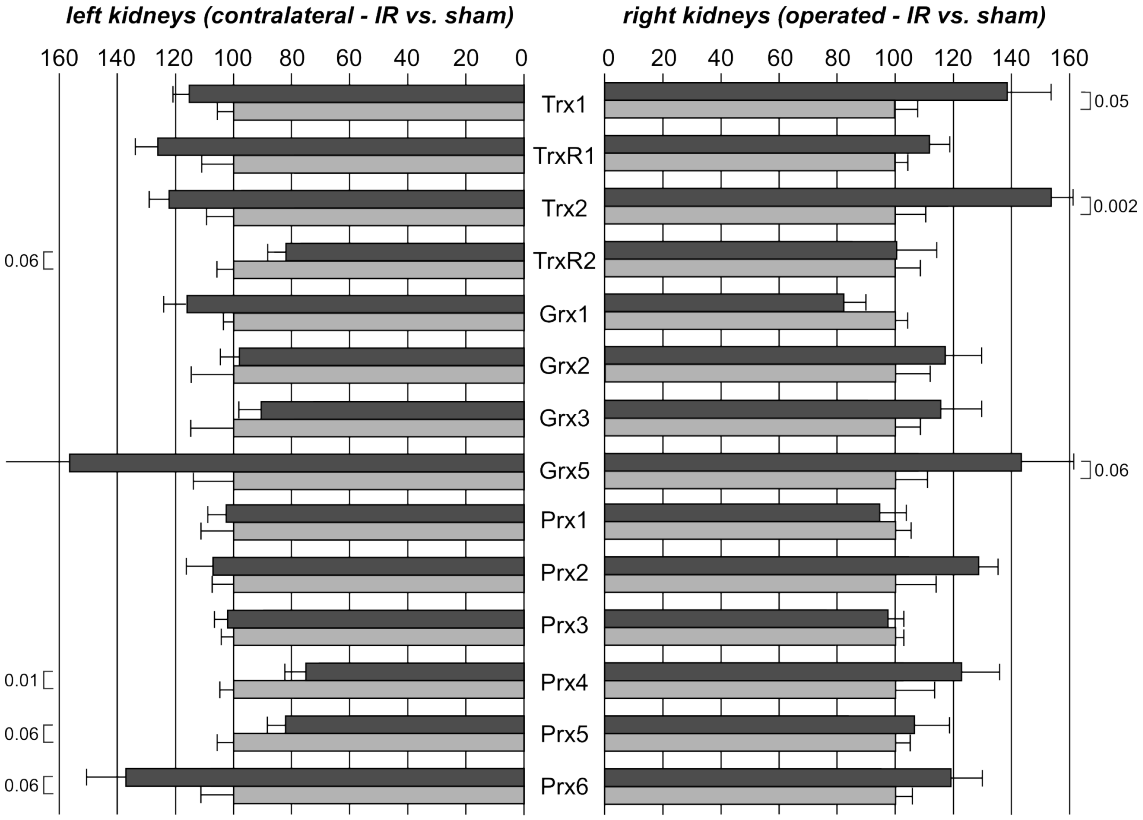

Figure 24: Expression levels of Trx family proteins in a model for renal ischemia/reperfusion injury. The I/R (right kidney), contralateral (left kidney) and sham kidneys were analysed for protein levels by Western blot and in the case of Grx2 by sandwich ELISA. Data were quantified using ImageJ and state the fold-increase in percent as means +/- SEM, comparing I/R and contralateral kidneys to the control sham kidneys (Godoy et al. 2011b, compare to 4.3).

Protein levels of members of the Trx family were analysed in the ischemic and the contralateral kidney by Western blot and ELISA. Figure 24 summarizes the results, representing the means +/- SEM, stating the fold-increase in percent of each protein of the I/R or the contralateral kidney with respect to the control sham operated kidneys. Most of the proteins did not show any significant changes, neither in the ischemic, nor in the contralateral kidney. However, the levels of Grx5, Trx1 and Trx2 were significantly increased to approximately 140-150 %, following the I/R insult. Grx1 was decreased to 82 %. In contralateral kidneys Grx5 was upregulated to more than 150 % and Prx6 to 138 %. TrxR2, Prx4 and Prx5 were downregulated compared to the control kidneys. Immunohistochemical analysis of the distribution of redoxins in distinct regions of the kidney revealed that Grx2, Prx3 and Prx6 were the only redoxins which were exclusively overexpressed in proximal tubule cells, a cell type which can regenerate after an I/R insult (Sutton et al. 2002), (Nony and Schnellmann, 2003). Members of the Trx family have been shown to affect the cell cycle, regulating proliferation, differentiation and apoptosis (Powis et al. 1998), (Lillig et al. 2004) (Enoksson et al. 2005). To analyse if this is generally also valid for hypoxic conditions, Grx2, Prx3 and Prx6 were overexpressed in HEK293 cells using pExpress plasmids (compare to Figure 10 and 3.1.3). HEK293 cells were chemically transfected using lipofectamin (described in 2.2.2.6.2). Six hours after transfection, cells were transferred into the hypoxic incubator at 0.1 % O_2 for 24 h, followed by a reoxygenation period at 20 % O_2 for another 24 h. The efficiency of chemical transfection was

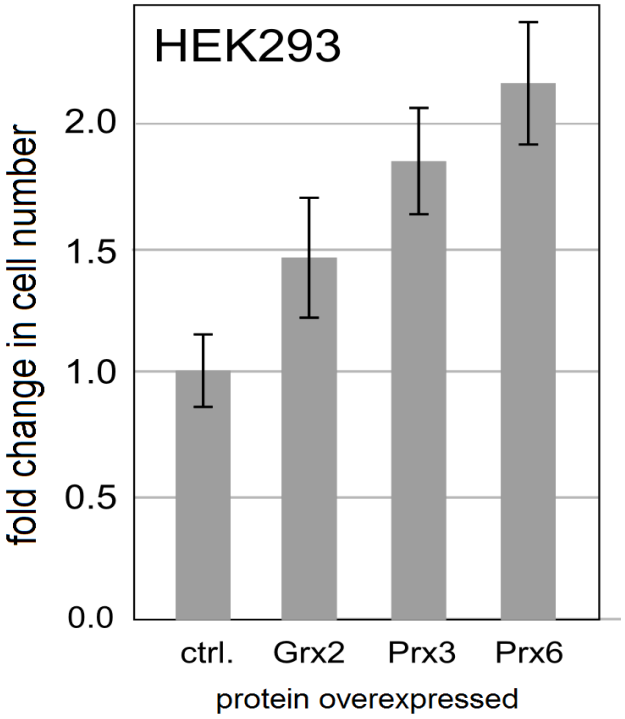

Figure 25: Overexpression of Grx2, Prx3 and Prx6 in HEK293 cells exposed to hypoxia. Cells were transiently transfected with redoxins and an empty plasmid as control (ctrl) using lipofectamin. Cells were incubated at 0.1 % O_2 for 24 h and reoxygenated at 20 % O_2 for another 24 h. The cell number was determined using an automatic cell counter. The average of ≥ 5 independent experiments is illustrated ± the standard error of the mean (adapted from Godoy et al. 2011b).

generally similar to the physical approach of electroporation, analysed by Western blot and ELISA, leading to a two to three fold overexpression, compared to control cells transfected with an empty pExpress plasmid (data not shown). Overexpression of all three redoxins in HEK293 cells led to an increase in cell number compared to control cells, analysed using an automatic cell counter (Cellometer, PeqLab). Figure 25 depicts the increase in cell number normalised to the control. Overexpression of the mitochondrial hGrx2 increased the cell number about 1.5 times, hPrx3 overexpression about 1.8 times and Prx6 more than two times.

3.6 Trx family proteins in a cellular model for pancreatic β-cell transplantation

We have used the murine cell line MIN6 (compare to 2.1.7.2) as cellular model to analyse the responses of pancreatic β-cells to changes in the environmental oxygen concentrations. MIN6 cells constitute a highly differentiated and glucose responsive cell line, which is widely used in the field of diabetes research. Transplantation of pancreatic β-cells is regarded as a promising therapeutic approach for diabetes mellitus. However, during the isolation and subsequent implantation, β-cells are exposed to different oxygen concentrations. The generation of ROS and inflammatory reactions may prevent longterm cell survival, regeneration of β-cells and restoration of insulin production (Chou and Sytwu, 2009). MIN6 cells were very sensitive towards hypoxic insults, compared to the analysed HEK293 cells. MIN6 cells were cultured for 12 h at 1 % and 2 % O_2 – an incubation time which led to 40 % cell death compared to cells incubated at 20 % O_2, measured with the proliferation and viability XTT kit (Roche, Mannheim, Germany) (data not shown). Part of the cultures were reoxygenated for 12 h at 20 % O_2. Medium was collected to analyse protein secretion, as well as cells, lysed in NP40-containing buffer, to determine GSH and protein levels. Total GSH levels were analysed in a colorimetric enzymatic assay measuring the formation of the yellow TNB from DTNB, using a 96-well plate reader (described in 2.2.3.10). Figure 26 illustrates that the intracellular GSH levels decreased upon the hypoxic insult in an oxygen-dependent way. Cells grown under 20 % O_2 contained 20 nmol/mg protein total GSH, whereas cells grown in an atmosphere containing 1 % or 2 % O_2 only had GSH levels of 4 nmol/mg protein and less than 5 nmol/mg protein, respectively. This effect was reversible, the levels increased again to 7 nmol/mg protein and 9 nmol/mg protein for

cells cultured at 1 % and 2 % oxygen, respectively. Grx2 levels were analysed using the established sandwich ELISA. Figure 26 shows that the intracellular Grx2 levels responded similar to the total GSH levels. Grx2 levels decreased upon low levels of oxygen. Cells cultured in an atmosphere containing 2 % oxygen contained 55 % Grx2, cells grown at 1 % oxygen 40 %, compared to cells grown under normoxic, i.e. 20 % O_2, conditions. Reoxygenation led to an increase in both cases with Grx2 levels recovering to approximately 80 %. The other redoxins were analysed by Western blot using 20 µg of total cell extracts, reduced with 100 mM DTT and 50 mM TCEP. Figure 26 summarizes the Western blots. The hypoxic insults led to distinct responses in the protein amount of the redoxins. Grx1, Grx3 and Grx5 diminished with lower O_2 concentrations. For Grx1 a higher expression was detected after the reoxygenation, compared to the hypoxic condition, whereas no change was seen for the monothiol Grxs Grx3 and Grx5. Prxs responded differently to the hypoxic insults. The protein amounts of Prx2 and Prx6 strongly decreased with less oxygen, while Prx4 levels increased. Prx3 was also downregulated upon hypoxia and even less protein was detected after the reoxygenation period. Cytosolic Prx1 and mitochondrial Prx5 showed similar changes, with strongly increased protein levels after hypoxia and decreased levels following the reoxygenation. The Trx system showed opposite responses to the growth conditions, when comparing the cytosolic and the mitochondrial

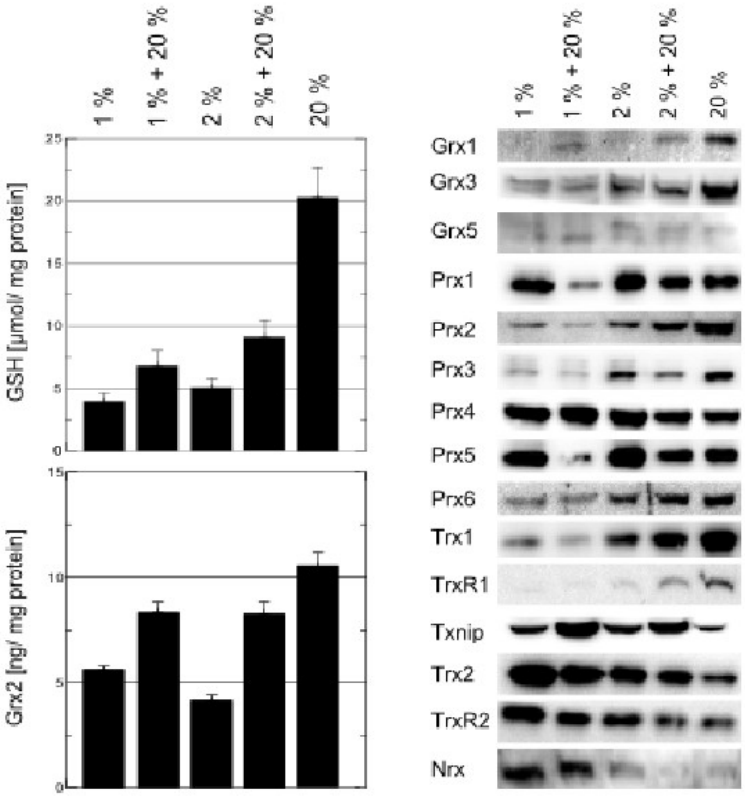

Figure 26: Total GSH levels and protein expression of Trx family proteins in MIN6 cells grown for 12 h at 1 %, 2 % or 20 % O_2, plus 12 h reoxygenation at 20 %, if indicated. Total GSH levels were measured in a colorimetric assay using DTNB as substrate. Protein levels were analysed by ELISA and Western blot.

systems. The levels of cytosolic Trx1 and TrxR1 were significantly decreased, whereas the levels of mitochondrial Trx2 and TrxR2 increased. The cytosolic/nuclear Nrx, which contains Trx and Grx domains, acted comparably to Trx2. Txnip, which was discovered as inhibitor of Trx, but was also shown to have functions independent from the oxidoreductase, was also upregulated, showing a higher expression after reoxygenation. Similar results were also seen after 24 h of reoxygenation (data not shown).

Various redoxins, including Grx1, Grx3, Trx1 and Nrx were described to translocate through the cell, with a change in localisation being essential for the diverse functions of the protein family. The cellular distribution of the redoxins was analysed by immunocytochemistry (described in 2.2.3.9). Besides a reduction in the general intensity of Trx1 staining, no significant changes in the subcellular localisation of the above mentioned proteins was detected (data not shown).

Moreover, the potential secretion of the redoxins was analysed in medium samples by performing Western blot analysis. Only Trx1 was detected in the medium of cells grown under hypoxic conditions and after reoxygenation. Within the above described experiments, cells were not washed between the hypoxic insult and the reoxygenation period. Therefore, secretion of Trx1 was furthermore analysed in cells, which received fresh medium between the two conditions. Trx1 was only observed in the medium after hypoxia, not after reoxygenation (Figure 27). The diverse effect on the cytosolic and the mitochondrial Trx system was also seen in primary islet cells derived from pig (compare to 2.1.7.2 and 2.1.8.3.1). However, the overall signals in the Western blots were weaker, compared to human, mouse or rat antigens (data not shown).

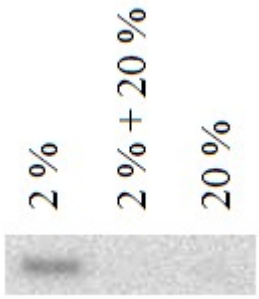

Figure 27: Secretion of Trx1 in MIN6 cells, cultured for 12 h at 20 % or 2 % O_2, plus 12 h reoxygenation at 20 %, if indicated.

In addition, the group of Prof. Dr. Linn at the Justus-Liebig Universität Gießen, confirmed our data in an animal model for diabetes or rather the transplantation of pancreatic β-cells. They analysed tissues of healthy, non-diabetic mice and mice with streptozotocin-induced diabetes (compare to 2.1.8.3) which received pancreatic β-cells via an injection into the portal vein. The injected cells settled in the liver and started producing insulin. Pancreas as well as the liver of transplanted animals were analysed 30 min (hypoxic state) and 120 days (reoxygenation state) after the transplantation by

performing immunofluorescence. Pancreatic β-cells were costained with an antibody against insulin. Trx1 and TrxR1 were downregulated whereas Trx2 and TrxR2 were upregulated in β-cells shortly after transplantation. After 120 days, no significant changes were detected, compared to untreated β-cells within the healthy pancreas (data not shown, compare to 4.3).

We furthermore explored if the overexpression of hTrx1 and hTrx2 in MIN6 cells affected cell survival after hypoxia and reoxygenation, analysing the phosphorylation pattern of the MAP kinases ERK1/2, JNK1/2/3 and p38. MAP kinases regulate cell proliferation, differentiation and apoptosis via phosphorylation cascades in response to for instance growth or stress stimuli.

MIN6 cells were incubated for different durations in an atmosphere containing 2 % O_2 or 20 % O_2. Upon normoxic conditions nearly no phosphorylated ERK1/2 was detected by Western blot. Increased levels of phosphorylated ERK1/2 were detected after six hours hypoxia, increased up to 12 h and were stable up to 24 h (data not shown).

MIN6 cells were transfected in 6-well plates using lipofectamin. After 12 h cells were transferred into the hypoxic chamber for 12 h in an atmosphere containing 2 % O_2. Part of the cells were reoxygenated for 24 h at 20 % O_2. Cells were harvested and lysed in NP40-containing lysis buffer supplemented with phosphatase inhibitors. MIN6 cells grown under normoxic conditions constituted the proliferative state. Cells cultured in a hypoxic environment reacted with an anti-stress response, whereas cells exposed to hypoxia and reoxygenation responded with an anti-apoptotic reaction. Trx1 overexpression slightly increased the phosphorylation of ERK1 and ERK2 under normoxic conditions, but not under hypoxic conditions. Trx2 overexpression significantly increased the phosphorylation of ERK1 and ERK2 under normoxic, but not under hypoxic conditions.

Increasing the intracellular levels of Trx1 furthermore decreased the levels of phosphorylated JNK1, JNK2 and JNK3 under hypoxia and decreased the amount of phosphorylated p38 under hypoxia and reoxygenation. Elevating the levels of Trx2 also led to lower levels of JNK1, JNK2 and JNK3 under hypoxia, but only decreased levels of phosphorylated p38 slightly under hypoxia and reoxygenation (Figure 28).

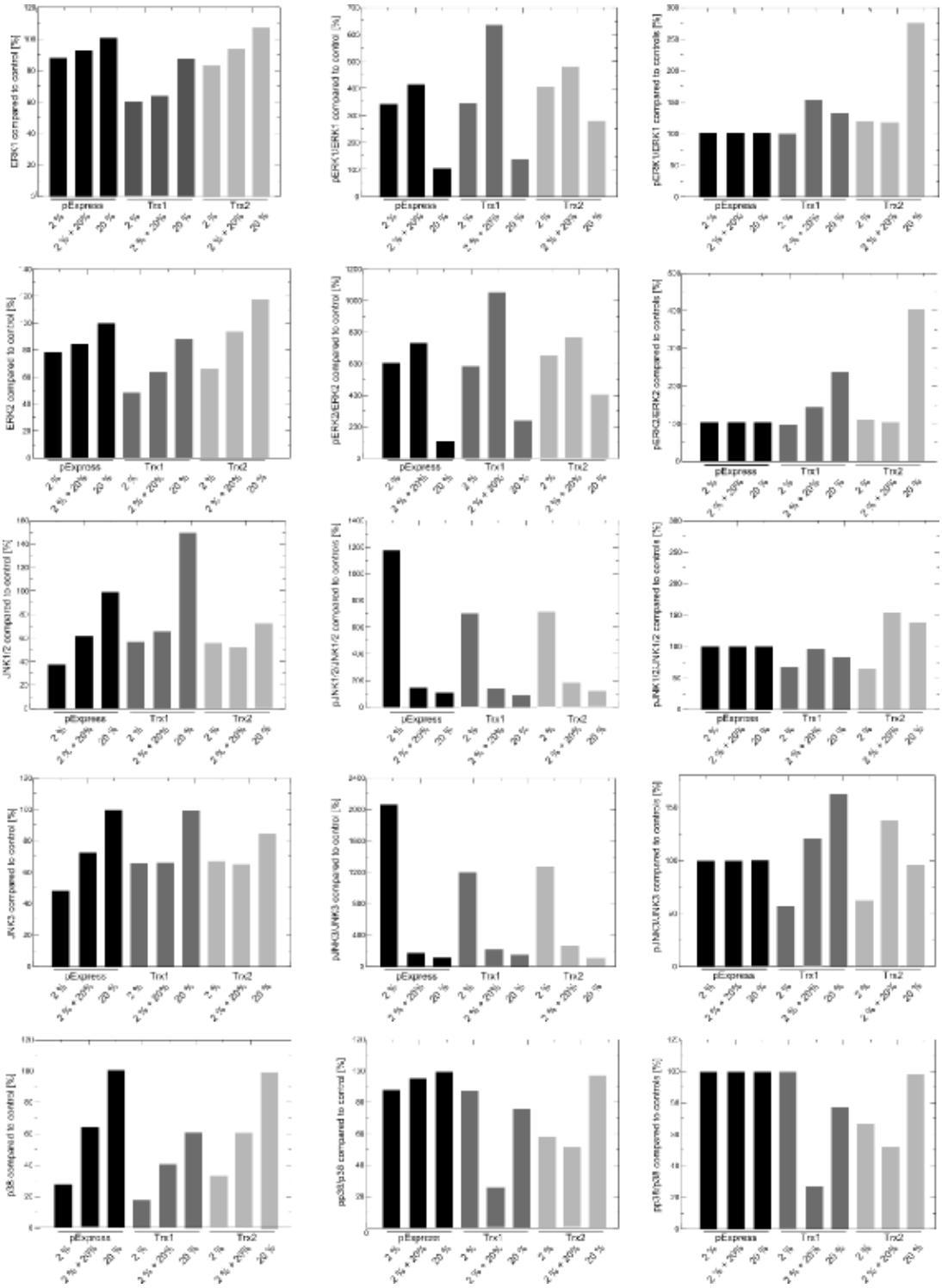

Figure 28: Phosphorylation of MAP kinases in MIN6 cells grown under hypoxic conditions. MIN6 cells were transfected with an empty pExpress plasmid, Trx1-pExpress and Trx2-pExpress and grown for 12 h at 2 % or 20 % O_2, followed by 24 h reoxygenation at 20 % O_2, if indicated. Cell lysates were analysed for levels of ERK1 (row 1), ERK2 (row 2), JNK1/2 (row 3), JNK3 (row 4) and p38 (row 5). Column 1 illustrates total protein levels in %, compared to pExpress control cells grown at 20 % O_2. Column 2 shows phosphorylated protein in %, compared to pExpress control cells grown at 20 % O_2. Column 3 displays phosphorylated protein in %, compared to pExpress control cells grown under each specific O_2 concentration.

4. Discussion

4.1 New substrates, new functions

The oxidoreductases of the glutaredoxin system reduce disulfides and catalyse the de-/glutathionylation in a wide range of substrates. Grxs were shown to reduce the catalytic disulfide of 2-Cys Prxs in plants (Rouhier et al. 2002).

In mammalian cells on the other hand, the reduction of 2-Cys Prxs was only ascribed to the Trx system (Chae et al. 1999). Cytosolic Grx1 was analysed in these studies with no detectable peroxidase activity for the cytosolic Prx1 and Prx2, as well as for the mitochondrial Prx3 and Prx5 (Seo et al. 2000). Mitochondrial Grx2 was not included. Since these studies, Grx2 has obtained a special status in the family, because it is able to receive electrons not only from GSH, but also from TrxR, combining the features of the Grx and the Trx system (Johansson et al. 2004). We have shown here for the first time, that mammalian Grx2 catalyses the reduction of the intermolecular disulfide of the 2-Cys Prx3. Silencing the expression of Grx2 or Trx2 in HeLa cells did not increase the level of oxidised dimeric Prx3, indicating that if, upon any stimuli one of the two systems is rendered inoperative or is downregulated, the other system can take over. *In vitro* kinetics of recombinantly expressed proteins, demonstrated that Grx2 can donate electrons to the 2-Cys Prx3, with similar reaction parameters as Trx2. Using TrxR2 as electron donor, H_2O_2 as substrate and following NADPH consumption, we showed that the catalytic efficiency of Grx2

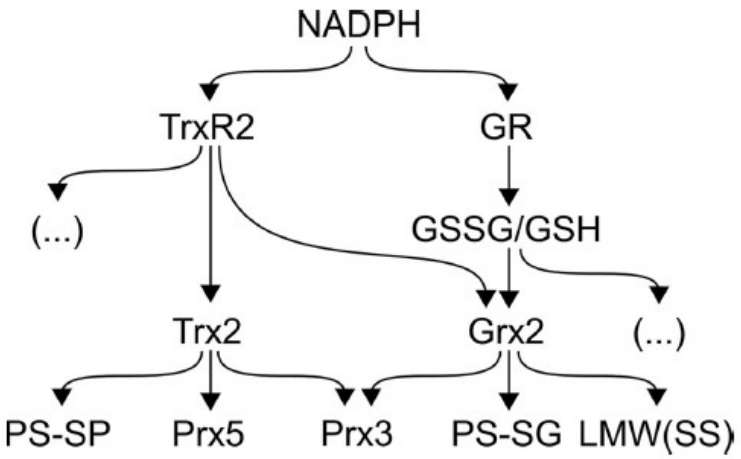

Figure 29: Electron flow of mitochondrial redoxins. NADPH reduces TrxR2, which among other substrates (…) reduces Trx2. Trx2 reduces protein disulfides (PS-SP), for instance of Prx3 and Prx5. NADPH also reduces GR, which donates electrons to GSH, which among other substrates (…) donates electrons to Grx2. Grx2 can also receive electrons from TrxR2. Grx2 reduces low molecular weight disulfides (LMW(SS)), GSH-mixed disulfides (PS-SG) and the 2-Cys Prx3 (Hanschmann et al. 2010).

corresponded to 55 % of the activity measured for Trx2 (Hanschmann et al. 2010). It is not clear if under physiological conditions both systems function equally as electron donor. Simultaneous silencing of the expression of both redoxins in HeLa cells led to the accumulation of up to 60 % oxidised Prx3. We excluded that the oxidation of Prx3 was the result of increased oxidising conditions due to the silenced expression of the two oxidoreductases, measuring protein carbonylation and glutathionylation.

The reason why Prx3 was not fully oxidised can be explained by the fact that the protein levels of Grx2 and Trx2 were only reduced to 5-20 % compared to scrambled-control siRNA or by the possibility of the existence of another electron donor.

Moreover, even though the 2-Cys Prx-specific redox blot constitutes a strong tool to distinguish between monomeric and dimeric Prxs, it cannot detect single Cys oxidations without subsequent dimerisation, due to only minor changes in the molecular weight. Cys residues can, for instance, be oxidised to sulfinic or sulfonic acid. Two modifications, which are generally irreversible. However, in the case of Prxs, these overoxidations, can be exclusively reduced by sulfiredoxins (Woo et al. 2005). The reduction of Prx3 by Grx2 was shown to depend on the dithiol mechanism, because the Grx2 Cys35Ser mutant, which lacks the C-terminal active site Cys and only supports the monothiol reaction mechanism, did not show any activity in the *in vitro* kinetics (Hanschmann et al. 2010). This finding generally excludes a function for the monothiol glutaredoxins as electron donors for 2-Cys Prxs, but does not explain why Grx1 cannot reduce the cytosolic 2-Cys Prxs (Seo et al. 2000). Grx2 contains the active site motif Cys-Ser-Tyr-Cys, enabling it to receive electrons from TrxR (Johansson et al. 2004) and to coordinate the [2Fe2S] cluster (Berndt et al. 2007). The Grx1 mutant protein (Pro23Ser), mimicking the active site motif of Grx2, was also able to coordinate the iron cofactor (Berndt et al. 2007). It is tempting to speculate that the mutant protein might also be able to reduce cytosolic 2-Cys Prxs. At the same time the Grx2 mutant protein with the active site motif of Grx1 should not be able to reduce mitochondrial Prx3. Moreover, cytosolic 2-Cys Prxs, which were identified as interaction partners of Grx2c in HeLa cells by intermediate trapping, might be substrates for the cytosolic/nuclear cancer/testis-specific isoform (Hanschmann et al. 2010).

An analysis of the distribution of the redoxins in tissues of the mouse by immunohistochemistry confirmed the interaction between Grx2 and Prx3. Different tissues clearly showed a distinct correlation between the redoxins TrxR2, Trx2, Grx2 and Prx3. Cells of the connective tissue displayed a strong staining for Trx2 and Prx3

without detectable expression of Grx2. In oviduct tissue on the other side the distribution of TrxR2, Grx2 and Prx3 correlated, while Trx2 was absent from the epithelial cells. In the endometrium of the uterus the expression of TrxR2 and Trx2 correlated as well as the distribution of Grx2 and Prx3 (Figure 30). Because Prxs act in regulating the levels of the signaling molecule hydrogen peroxide, these data point to an individual, cell type-specific contribution to signaling pathways of the different redoxins.

The second mitochondrial Prx was also included in our studies, revealing that the reaction cycle of the atypical 2-Cys Prx5 only relied on the Trx system *in vitro* (compare to Figure 29). Prx5 can be glutathionylated (Fratelli et al. 2003), a posttranslational modification that is generally efficiently catalysed by Grxs. So far, the de-glutathionylation of human Prxs is only ascribed to sulfiredoxins (Park et al. 2009). As discussed above, this modification could not be detected using the 2-Cys redox blot. We demonstrated that Prx3 was rapidly oxidised and also recovered in H_2O_2 treated HeLa cells, emphasizing that redox signaling is a rapid and specific form of protein regulation. One could not distinguish untreated HeLa cells from cells, treated with 50 µM H_2O_2 for 30 min, because the oxidised Prx3 was already recovered (Figure 18). Therefore, it is important to run kinetics when analysing redox signaling processes to exclude the source of error "incubationtime".

The shown kinetic constitutes one example for the specific oxidation and reduction of one protein, hPrx3, by one oxidising compound H_2O_2 with one fixed concentration of 50 µM in one cell line and should not be translated on other proteins, other oxidising agents or other cell lines. We have analysed the oxidation/reduction of hPrx3 in different cell lines using different concentrations of different compounds including phenylarsine oxide (inhibition of dithiols), doxorubicin (ROS induction, oxidative modifications of DNA) and terbutylhydroperoxide (RNS induction, oxidative modifications of lipids) for different incubation times. Due to the limitations of the 2-Cys Prx-specific redox blot, we were not able to analyse the "overall" redox state of the peroxidase. However, the general monomer-dimer ratio drastically differed between the cell lines (data not shown), implying distinct levels of Trx family proteins, but also the fact that proteins might be differently affected and implicated in the cellular response to various conditions.

Indeed, the field of redox-biochemistry lacks methods which allow the analysis of the redox state of any protein *in vivo*, in general and the time-resolved analysis in specific.

Part of these results was published as Hanschmann *et al.* 2010 in the Journal of Biological Chemistry with the title “Both thioredoxin 2 and glutaredoxin 2 contribute to the redox state of mitochondrial 2-Cys Prx3” (Hanschmann et al. 2010). Figure 29 summarizes the electron flow of mitochondrial redoxins.

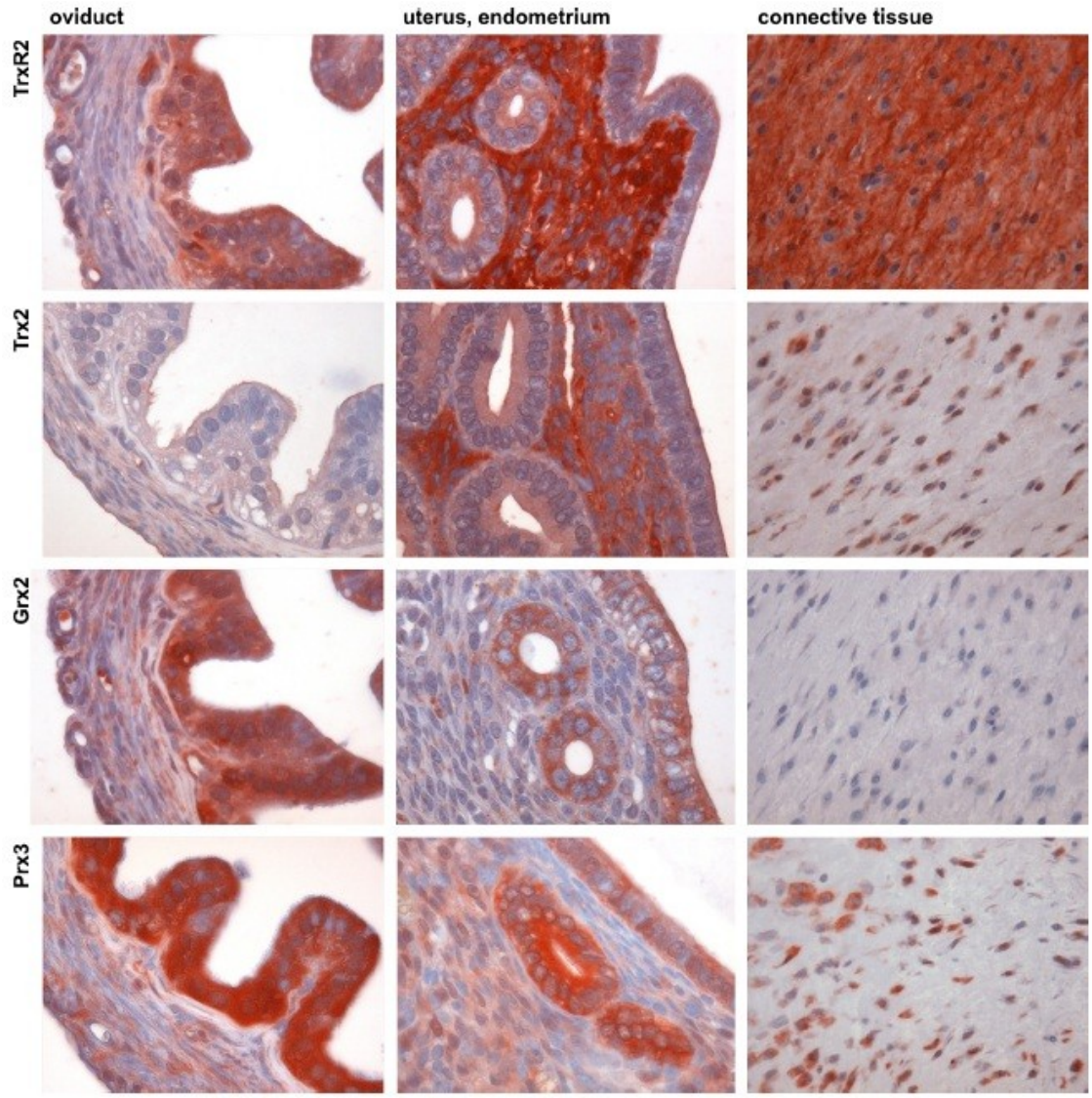

Figure 30: Distribution of TrxR2, Trx2, Grx2 and Prx3 in mouse oviduct, uterus and connective tissue. Strong staining for TrxR2, Grx2 and Prx3 was observed in oviduct tissue (magnification: 500x), while Trx2 was absent. The endometrium of the uterus (magnification: 500x) showed immunoreactivity for Grx2 and Prx3, as well as TrxR2 and Trx2. A strong staining for Trx2 and Prx3, but no staining for Grx2 was detected in the connective tissue (Hanschmann et al. 2010).

Human Grx2 was identified as the first member of the Trx family complexing a FeS cluster (Lillig et al. 2005). Lee *et al.* demonstrated that inhibition of mitochondrial Grx2 by GSH depletion resulted in mitochondrial dysfunction and iron overload, features of

neurodegenerative disorders such as Parkinson´s disease (Lee et al. 2009). Grxs were shown to be required for iron homeostasis and FeS cluster biosynthesis. It has been proposed that this function is related to the ability to coordinate a [2Fe2S] cofactor. Recent studies indicated potential functions as iron sensors, scaffold proteins for the synthesis of FeS clusters or as transporters, transferring the cofactor to apo-proteins (reviewed in Rouhier et al. 2010).

The GPAT levels in Grx2 depleted cells were decreased to 60 %, suggesting potential functions in FeS cluster biosynthesis. Grx5, the second mitochondrial Grx has been implicated in FeS cluster assembly in various organisms. Yeast mutants, which lack the monothiol Grx5 were characterised by iron overload and the accumulation of inactivated FeS proteins (Mühlenhoff et al. 2010). Loss of Grx5 in the zebrafish mutant *shiraz* and a human patient with decreased Grx5 expression due to a mutation in an intronic region of the *GRX5* gene showed hypochromic anemia, which is linked to impaired hemoglobin synthesis in erythrocytes (Camaschella et al. 2007), (Wingert et al. 2005). Recent studies showed that recombinantly expressed bacterial, yeast, plant and human Grx5 orthologues can coordinate labile GSH-ligated [2Fe2S] cofactors (Bandyopadhyay et al. 2008), (Picciocchi et al. 2007), suggesting a potential function as scaffold protein in FeS cluster biosynthesis. Comparably to Grx5, hGrx2 contains a labile FeS cluster, implying a potential role as scaffold protein. The [2Fe2S] cofactor is coordinated by the N-terminal active site Cys from two Grx2 monomers and two GSH molecules (Berndt et al. 2007). Grx2 was proposed to act as redox sensor, because the iron cofactor is lost when the levels of reduced GSH decrease, leading to monomerization and activation of the oxidoreductase (Berndt et al. 2007). A similar mechanism could be the case upon iron deprivation. In yeast, the cytosolic monothiol Grx3 and Grx4 form a stable [2Fe2S]-bridged complex. Mühlenhoff and coworkers demonstrated that Grx3/4 act in iron trafficking and iron sensing, two functions which depended on the iron cofactor. Depletion of both proteins in yeast impaired the synthesis of heme- and FeS proteins. Even though cytosolic levels of iron were comparably high, iron could not be transferred to the mitochondria (Mühlenhoff et al. 2010). Besides Grx3/Grx4 two other proteins were shown to function in the cellular transport of iron. Human poly (rC)-binding protein 1 (PCBP1) was shown to bind iron and load the redox-reactive metal onto the storage protein ferritin. Knock-down of PCBP1 in the human hepatoma cell line HuH7 inhibited iron storage by ferritin and increased the cytosolic free iron pool (Shi et al. 2008).

Depletion of lipocalin 24p3/2,5-DHBA, a protein binding to siderophores, led to cytosolic iron overload and mitochondrial iron deficiency.

Grx2 depleted HeLa cells did not show a comprehensibly impaired iron regulation, even though ferritin levels were significantly decreased. However, only IRP1 was analysed, not IRP2. Cells lacking IRP1 showed a regular iron regulation, conducted by IRP2 (Schalinske et al. 1997), which generally exists in lower intracellular concentrations (Henderson, 1996) and is not regulated via an iron cofactor. Recalcati and collegues demonstrated that IRP1 and IRP2 responded in the opposite way to NO-induction in the murine macrophage cell line J774 and that IRP2-mediated posttranscriptional regulation is essential for controling the cellular levels of ferritin. Following the treatment with interferon γ/LPS, IRP1 activity was slightly increased, whereas IRP2 activity was reduced, leading to enhanced synthesis of ferritin (Recalcati et al. 1998). Grx2 depletion slightly decreased the levels of IRP1 and reduced the levels of ferritin to nearly 30 %, compared to control cells. It is tempting to speculate that IRP2 expression might be elevated in these cells.

In the presence of the signaling molecule NO, IRP1 was shown to lose its iron cofactor (Drapier et al. 1993), explaining the above described increase in protein activity in J774 cells. This effect is furthermore enhanced in the presence of reduced Trx1. However, Trx1 was shown to have nearly no effect on IRP1 by itself. The authors suggested that NO does not only disrupt the cluster but also oxidises the residues Cys^{503}, Cys^{506} and Cys^{437}, essential for cluster coordination. Reduced Trx1 then reduces the potential disulfide bond between two of the thiol groups of IRP1, restoring the mRNA binding activity (Oliveira et al. 1999). Even though IRP2 does not coordinate an iron cofactor it possesses the three cysteine residues, as well as another five thiol groups in its specific insertion sequence, which is essential for protein degradation upon iron overload (Iwai et al. 1995). IRP2 was inhibited by peroxinitrite and activated by the reducing agent β-mercaptoethanol (Bouton et al. 1997). Oliveira *et al.* also demonstrated that NO donors inhibit the mRNA capacity of IRP2, which can be restored by reduced Trx1, but not by GSH (Oliveira et al. 1999).

Grx3 depletion in HeLa cells displayed a strong effect on iron homeostasis, impairing cytosolic and mitochondrial heme- and FeS cluster containing proteins. Cells exhibited features of cellular iron deprivation, even though the general iron supply and uptake of iron was not affected, as measured by Fe^{55} incorporation and in a colorimetric assay according to Fish (compare to Haunhorst *et al.* 2011, unpublished manuscript, 4.3).

Grx3 shares the active site motif of Grx5. It is composed of one Trx- and two monothiol Grx domains, with each Grx domain coordinating a [2Fe2S] cluster. Like Grx2 and Grx5 the cofactor is coordinated by the active site Cys of two monomers and two covalently bound GSH molecules (Haunhorst et al. 2010).

The Grx3-depletion phenotype described in this study is similar to what was shown for Grx3/Grx4 in yeast, referred to above.

Knock-down in zebrafish, provided strong evidence that the function of Grx3 in cytosolic iron trafficking is conserved in vertebrates. The above described data, as well as the following analysis in zebrafish, are part of the manuscript Haunhorst *et al.* 2011 with the title "Glutaredoxin 3 (PICOT) functions in iron homeostasis and is essential for hemoglobin maturation in zebrafish." (compare to 4.3).

The model organism *Danio rerio* is commonly used for studying vertebrate development and due to the use of the morpholino technique, for the analysis of gene function (Lieschke and Currie, 2007). The zfGrx3 contains a Trx domain and two monothiol Grx domains, sharing 59 % sequence homology with the human Grx3 and 37 % with yeast Grx3 and Grx4. It is expressed in the CNS, heart and intermediate cell mass, constituting the region of embryonic erythropoiesis, detected by *in situ* hybridisation (Figure 31A). Grx3 knock-down was induced by injection of a specific morpholino directed against the translation initiation codon of zfGrx3 into one cell stage embryos. Grx3 depleted embryos, 48 hours post fertilisation (hpf), displayed impaired maturation of hemoglobin and reduced activity of iron-dependent proteins. Approximately 50 % of Grx3 depleted embryos lacked mature hemoglobin (Figure 31 B-E). This phenotype was rescued by coinjection with 40 pg zfGrx3 capped mRNA per embryo (Figure 31F).

In addition, cell extracts of whole wildtype and Grx3 knock-down embryos were analysed for enzymatic activities of total aconitase and cytochrome *c* oxidase, demonstrating a reduction to approximately 80 %, similar to what we have seen in HeLa cells. Malate dehydrogenase was measured as internal control (Figure 31G).

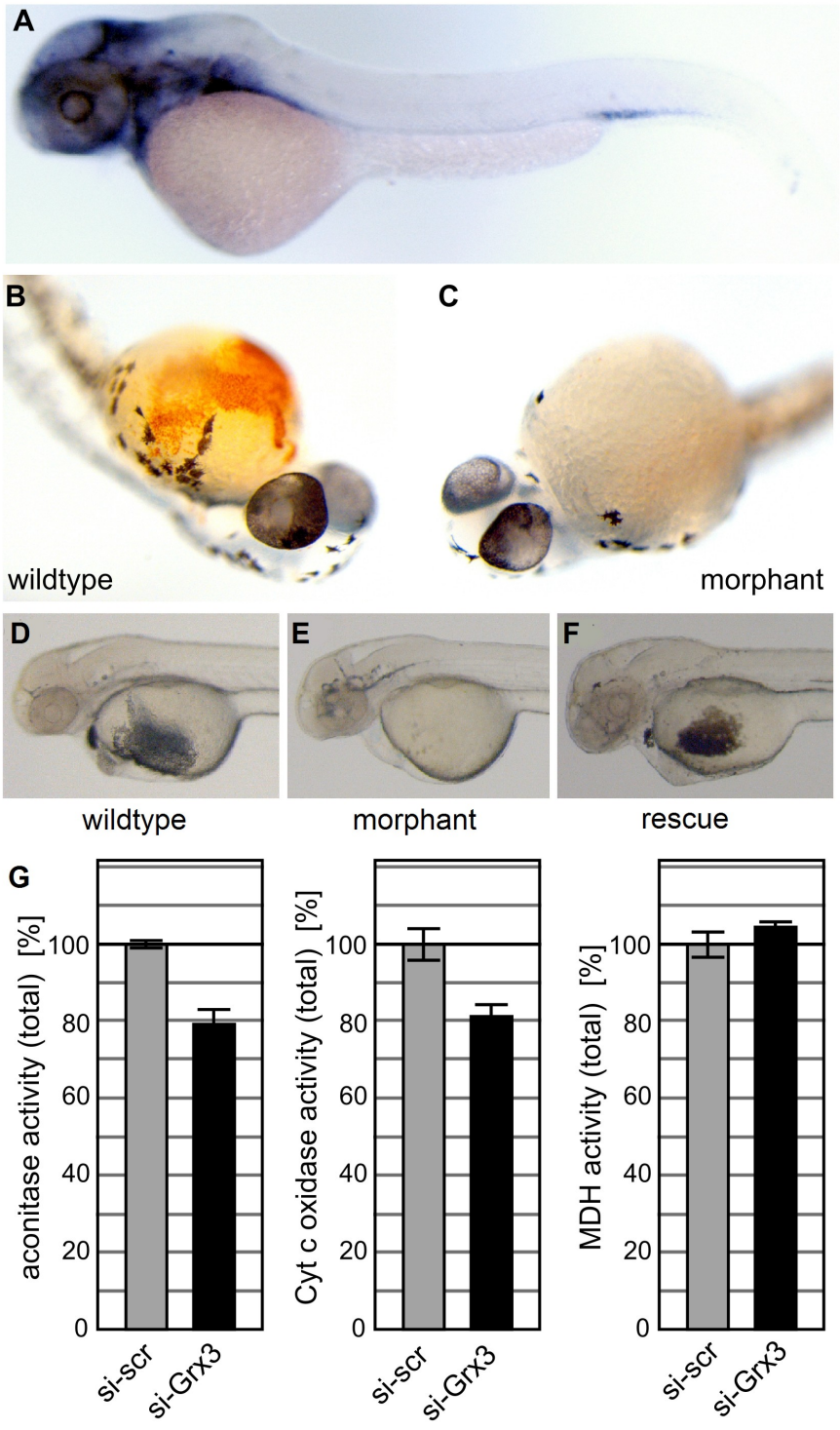

Figure 31: Grx3 depletion in 48 hpf zebrafish embryos impaired hemoglobin maturation and the activity of iron-related proteins. Localisation of Grx3 detected by *in situ* hybridisation (A). Hemoglobin staining by o-dianisidine in a WT (B) and Grx3-depleted (morphant) embryo (C). Hemoglobin staining by diaminofluorene in WT (D), morphant (E) and morphant embryo, rescued by coinjection with zfGrx3 capped mRNA (F). Enzyme activity measured in whole embryos: aconitase activity, cytochrome *c* oxidase and malate dehydrogenase as control (Haunhorst et al. 2011, unpublished manuscript, compare to 4.3).

4.2 Trx proteins in physiology and pathology

Even though it is important to gain information about single proteins of the Trx family, it is also desirable to understand how these protein networks respond to different stimuli or environmental changes. Because these proteins work in a network with different systems being connected at distinct points, we investigated 16 proteins of the Trx family in physiological and pathological conditions. The so called “Redox Atlas of the rat brain”, published as Aon-Bertolino et al. 2011 with the title “Thioredoxin and Glutaredoxin system proteins-immunolocalization in the rat central nervous system” in the journal Biochimica et Biophysica Acta, was an approach to map these proteins under physiological conditions. Together with the “Redox Atlas of the Mouse”, published by the AG Lillig (Godoy et al. 2011a) (compare to Figure 35) and the analysis of data of redoxins from the Human Protein Atlas (Dammeyer and Arnér, 2011), this study constitutes a data collection of the distribution of Trx family proteins, potential colocalisation of proteins and new functions, achieved by immunohistochemistry. For this approach specific antibodies were used, which were extensively evaluated for rat by Western blot. All tested antibodies displayed specific bands at the estimated sizes. Despite this validations, it is important to take the possibility of unspecific and false-negative staining into account. These problems can arise from tissue processing, embedding, fixation and the method of staining as well as general differences in the sensitivities of different antibodies. IHC allows a qualitative analysis of the distribution of a protein, but does not constitute a quantitative method to compare between different antigens (Godoy et al. 2011a) (Aon-Bertolino et al. 2011). The redox proteins were detected throughout the rat CNS. Golgi I type neurons showed the most consistent immunostaining in all areas analysed. The thioredoxin system (Trx1, TrxR1, Trx2, TrxR2) showed an intense staining for neurons and neuroglia in most regions of the CNS. These proteins showed a low staining in the hippocampus, an area of the brain which is extremely vulnerable towards oxidative stress. The staining for Txnip coincided with the staining of Trx1 with the exception for the spinal cord, where no staining for Txnip was detectable. Grx1 seems to be localised in nuclei of Purkinje cells and spinal cord motoneurons. Grx1 displayed a strikingly intense immunostaining in glia cells in the striatum radiatum. Grx2 was strongly labeled in type I neurons and glia cells of all areas investigated. Grx3 and Grx5 displayed a staining

pattern in neurons of the spinal cord. A potential nuclear localisation was suggested. γGCS showed a strong staining in neurons and glia cells in hippocampus and cerebellum and a weak staining in neuroglia in all of the areas studied. Prx1 was strongly expressed in neurons, whereas astrocytes only showed a weak staining. Prx2 showed a strong staining in projection neurons. A similar pattern was seen in Golgi type I neurons for Prx3, Prx4, Prx5 and Prx6. Prx6 was furthermore shown to be localized in neuroglia. All data are summarized in Table 8. Figure 32 shows exemplary the staining for the Trx and Grx systems in the hippocampus.

Protein	***Cere-bellum***	***Hippo-campus***	***Striatum***	***Cortex***	***Substantia nigra***	***Spinal cord***	***Retina***
Trx1	++	+/-	+ (axons)	++	++	++	++
Trx2	+	+	++	+	++	+	+
TrxR1	+	+	+	+/-	+/-	-	+/-
TrxR2	++	+	+/-	+	+	+	(nucleus)
Txnip	+/-	+/-	+/-	+/-	+/-	-	+
Prx1	+	+	+	++	+	+ (nucleus)	+/-
Prx2	++	++	+	++	+	+	++
Prx3	+	+	+	++		+	+ (nucleus)
Prx4	+	++	+	++	+	++	+
Prx5	+	++	+	+	+	++ (nucleus)	+ (nucleus)
Prx6	++	++	++	++	++	+	+
Grx1	+ (nucleus)	++	+	++	+	++ (nucleus)	+ (nucleus)
Grx2	+ (nucleus)	+	++ (nucleus)	+	++	++	+
Grx3	+	+	++ (axons)	+	+	+ (nucleus)	+/- (nucleus)
Grx5	+	+	++	+	++	+ (nucleus)	+
γGCS	+	+	+	+	+	+	+/-

Table 8: Summary of the distribution of 16 Trx family proteins in different parts of the CNS: cerebellum, hippocampus, striatum, cortex, substantia nigra, spinal cord, retina (compare to Aon-Bertolino et al. 2011).

This data collection displays a tissue- and cell type-specific expression pattern, indicating distinct protein functions and complex crosstalk between the family members.

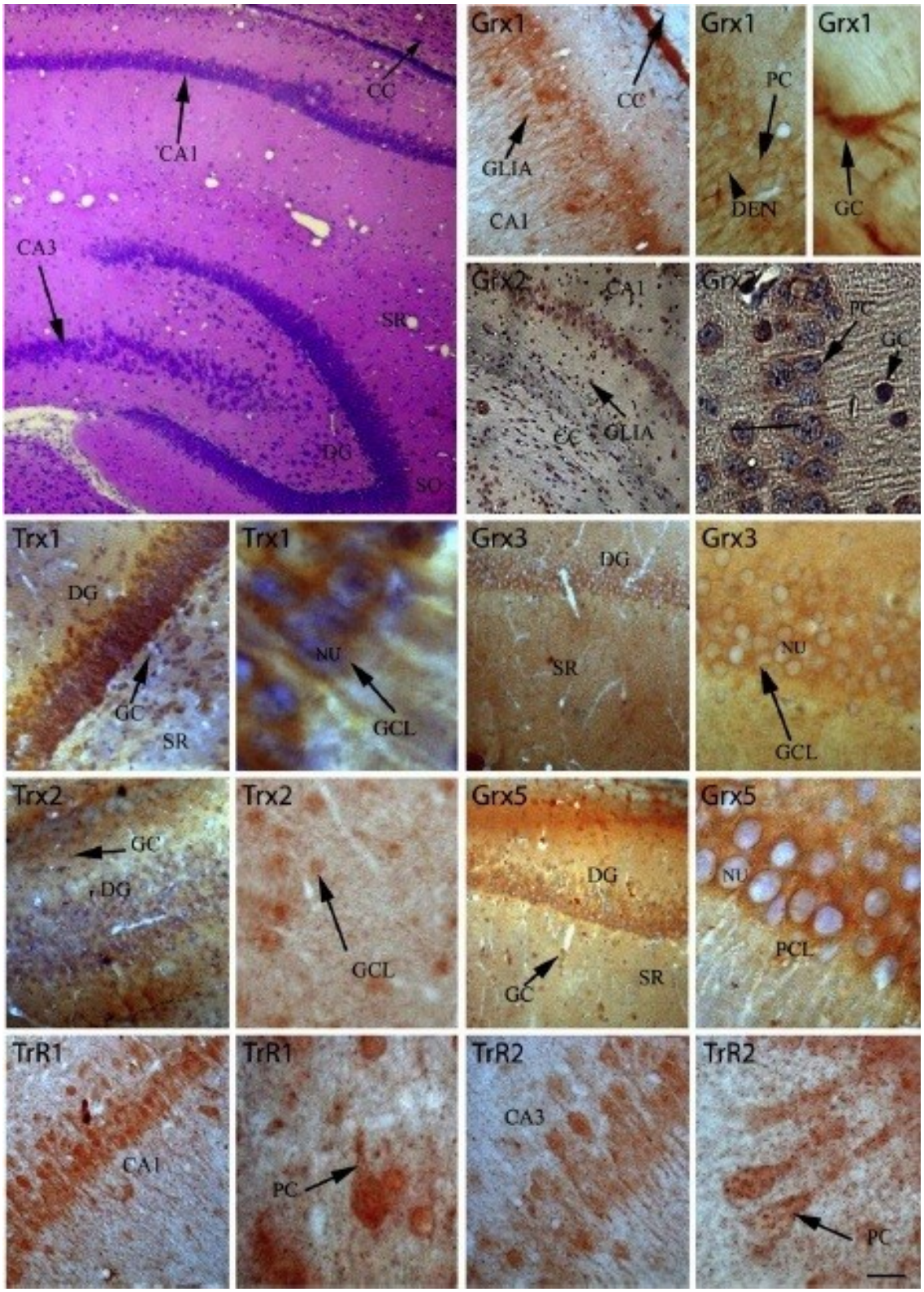

Figure 32: Immunohistochemical localization of Thioredoxin proteins in the rat hippocampus. Defined layers are shown in a hematoxilin-eosin stained slice (upper left corner). CC: corpus callosum, DG: dentate gyrus, PC: pyramidal cell, GC: glia cell, GCL: granular cell layer, DEN: dendrites, NU: nucleus. Scale bar: 10 µM (Aon-Bertolino et al. 2011).

The collected physiological data on the Trx family proteins were compared to samples, collected from rat pubs, suffering from perinatal asphyxia. The animal model was implemented in the group of Francisco Capani and constitutes a generally accepted and well established model.

Grx2, Trx1 and Trx2 were upregulated in the hippocampus 4 hours after the hypoxic insult. Together with Grx1, which was also analysed, but showed no detectable changes in expression or distribution, these proteins constitute the main cellular oxidoreductases in the cytosol and in mitochondria. These redoxins seem to be essential in the immediate response. However, 7 days after the hypoxic insult, the levels were back to normal. To understand the immediate, but also the longterm effects, triggered by the hypoxic insult, it is important to analyse all redoxins in the complete CNS. So far, no studies on Trx family proteins in perinatal asphyxia have been published. However, release of NO and ROS have been implicated in the disease, potentially leading to the disregulation of redox signaling, which could be responsible for the induction of apoptosis in the CNS and the longterm neurological deficits of the patients. Similar to the pathology of perinatal asphyxia, the disruption of redox control or generally the generation of ROS (Bonventre and Weinberg, 2003), (Wardle, 2005) by uncoupling of the mitochondrial chain (Baines, 2009) and inflammation (Weight et al. 1996), (Hutchens et al. 2008) was described as the most destructive cause of I/R injury. We have studied the effects of renal I/R injury on the Trx family using a mouse model, established in the AG Lillig. The pedicle of the right kidney was clamped for 30 min. The clamp was removed, the restoration of the blood flow was observed and the animals were sacrificed after 24 h.

Blood and urine samples of sham-operated and I/R mice were analysed (Table 9). Alterations in the blood cell count of the I/R group were detected, as well as an increase of segmented nuclei cells, indicating the beginning of an inflammatory response. Following I/R, urea elimination was significantly decreased and proteinuria, especially of high molecular weight proteins like albumin (Figure 33g) was observed.

The histological analysis of I/R kidneys showed typical changes after I/R injury i.e. tubular cell flattening, loss of brush-borders and luminal obstruction, compared to contralateral and sham-operated organs (Figure 33a-f). All these alterations constitute common changes after an I/R insult and demonstrated that the induction of renal I/R was successful (Godoy et al. 2011b).

Parameter	*Sham animals*	*I/R animals*	*p-values*
Body weight (g)	22.08 +/- 0.33	22.74 +/- 0.22	0.11
Urine protein (mg/ml)	47.73 +/- 11.1	62.24 +/- 6.8	0.26
Urea (mM)	8.47 +/- 0.51	10.8 +/- 0.72	0.04
Creatinine (µM)	14.27 +/- 0.77	14.51 +/- 0.84	0.83
Erythrocytes (T/L)	9.72 +/- 0.19	8.99 +/- 1.32	0.66
Hematocrit (L/L)	0.53 +/- 0.02	0.59 +/- 0.01	0.28
Hemoglobin (g/L)	150 +/- 3.65	165 +/- 5.35	0.06
Leukocytes (g/L)	7.17 +/- 0.7	7.5 +/- 0.33	0.66
Segmented nuclei (G/L; %)	1.48 +/- 0.11 (20.83 +/- 0.79)	3.23 +/- 0.24 (43.25 +/- 2.92)	0.0001
Lymphocytes (G/L; %)	4.78 +/- 0.55 (66.5 +/- 2.26)	3.35 +/- 0.32 (44.38 +/- 3.49)	0.0001
Monocytes (G/L; %)	0.75 +/- 0.13 (10.33 +/- 1.84)	0.83 +/- 0.14 (11.38 +/- 1.87)	0.69
Eosinophiles (G/L; %)	0.17 +/- 0.03 (2.33 +/- 0.33)	0.08 +/- 0.0 (0.88 +/- 0.353)	0.01
Basophiles (G/L; %)	n.d.	n.d.	-
Band neutrophiles (G/L; %)	n.d.	0.01 +/- 0.01 (0.13 +/- 0.13)	-

Table 9: Parameters for the successful induction of I/R. Physiological and blood parameters of sham-operated and I/R mice were determined by Laboklin (Bad Kissingen, Germany). The data stated in parentheses are derived from differential blood count; n.d.: not detected. Data represent the means +/- SEM of six sham-operated and 8 I/R animals (Godoy et al. 2011b).

Our results were published as Godoy *et. al.* 2011 in the Journal for Free Radical Biology and Medicine with the title “Segment-specific overexpression of redoxins after renal ischemia and reperfusion: protective roles of glutaredoxin 2, peroxiredoxin 3 and peroxiredoxin 6”. Analysing the expression levels of 15 Trx family proteins, we have seen significant changes in the ischemic kidneys i.e. an increase in the levels of Trx1, Trx2, and Grx5 and a decrease of Grx1 levels. In a previous study, Kasuno and coworkers demonstrated that Grx1 levels were generally higher in the cortex than in the medulla of the kidney, with no significant changes detectable after I/R (Kasuno et al. 2003). Grx1 is a protein which was found to be secreted from cells. Lundberg *et al.* detected the oxidoreductase in human plasma and demonstrated that Grx1 was secreted by unstimulated peripheral blood mononuclear cells, suggesting potential extracellular functions (Lundberg et al. 2004). Peltoniemi *et al.* showed that alveolar macrophages expressed Grx1 and that Grx1 levels were decreased in homogenates of the lung and in-

creased in the sputum of patients with chronic obstructive pulmonary disease, with the levels correlating to the stage of the disease and lung function (Peltoniemi et al. 2006). We detected an accumulation of the protein at the apical site of distal convoluted cells, but not in sloughed cells, and the specific secretion into the urine following I/R (data not shown). To our knowledge, this has not been described in any other study before. However, no extra-cellular functions for Grx1 have been demonstrated, so far. We believe that Grx1 could be used as a marker protein for renal I/R injury. Other redoxins, including Trx1, TrxR2, Prx2, Prx4 and Grx3 were detected in the lumina of tubules, but not in urine samples. Kasuno *et al.* have also detected Trx1 in the lumen of proximal tubules, but also in the urine (Kasuno et al. 2003). Trx1 was shown to be secreted by various cell lines (Rubartelli et al. 1992) and was detected in blood plasma in various disorders (Nakamura et al. 1996), (Jikimoto et al. 2002), (Kakisaka et al. 2002), (Nakamura et al. 2006). Extracellular Trx1 has been implicated in the attraction and activation of immune cells (Bertini et al. 1999). The cytosolic Grx3 was also shown to be secreted from cells and was implied in the modulation of the inflammatory response (Kato et al. 2008). However, part of the staining was also detected in sloughed cells, implicating

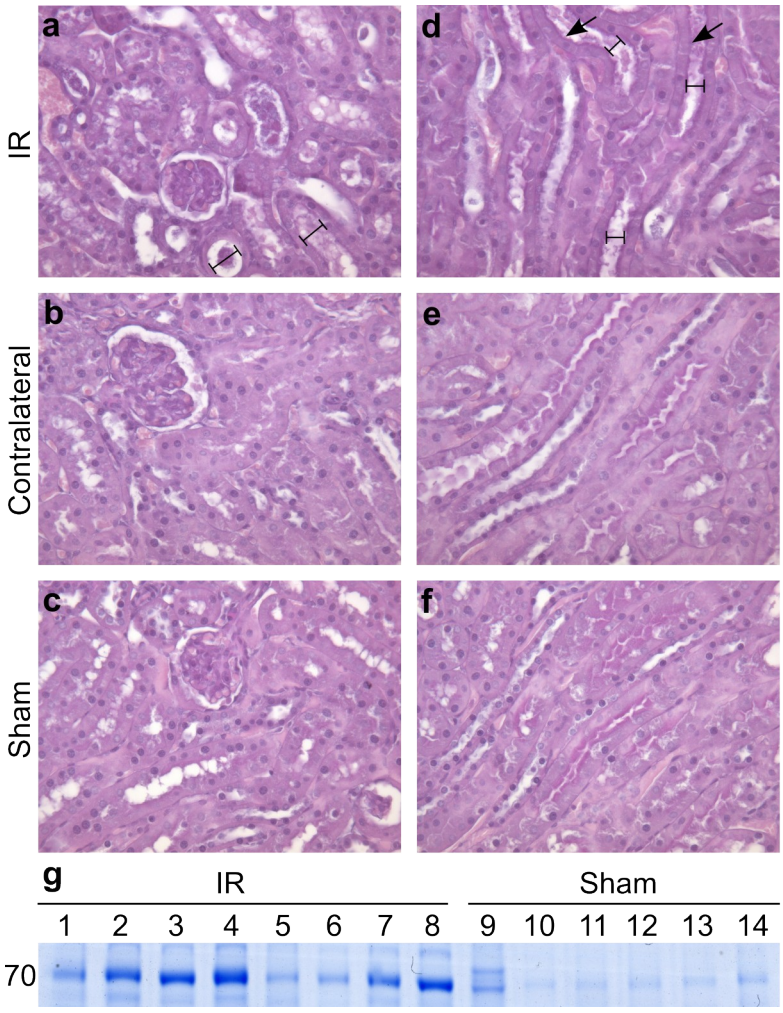

Figure 33: Parameters for the successfull induction of I/R. Histological analysis of sham, contralateral and I/R kidneys, i.e. cortex (a-c) and outer medulla (d-f) by Schiff´s periodic staining. In I/R kidneys epithelial flattening (dimension lines) and loss of brush borders (arrows) were detected. High molecular weight proteins in urine samples of sham and I/R animals (g).

that the presence of these redoxins might be due to non-specific processes. Besides the translocation of Trx1 from the cytosol into the extracellular space, Trx1 was also found in the nucleus, where it is known to regulate the transcription of various target proteins. We detected Trx1 in the nuclei of proximal tubule cells. This has also been shown in the study of Kasuno and coworkers. In the contralateral kidneys we have seen changes in the expression of proteins, too, indicating that the I/R does not only lead to changes within the affected organ, but also to systemic effects. An increase in the levels of Prx6 and Grx5 as well as a decrease of TrxR2, Prx4 and Prx5 was detected. Apart from protein levels, we were interested in analysing the distribution of redoxins throughout the nephron. Figure 34 summarizes the data, depicting the segments of the nephron and the distribution of the proteins, comparing ischemic and sham-operated organs.

We detected a segment-specific distribution of the proteins under physiological conditions. Furthermore, the response to the I/R insult was different in distinct parts of the nephron, implying tissue- or even cell type-specific functions of the Trx family proteins. We believe that these differences contribute to the distinct susceptibilities of different parts of the nephron towards the I/R insult. The glomeruli and the inner medulla cells for instance are relatively resistant, whereas medullary thick limb cells are especially susceptible to hypoxia (Hutchens et al. 2008), (Gobe and Johnson, 2007). Transgenic mice, overexpressing Trx1, were characterised by attenuated reperfusion-induced mTAL injury (Kasuno et al. 2003). The authors state that Trx1 was mainly observed in the cytosol of mTALs and decreased in other parts of the kidney, directly after the I/R insult. We confirmed the increased expression of Trx1 and furthermore detected Trx2 in mTALs using the specific marker THP (data not shown).

In contrast, we observed enhanced expression levels of Trx1 in the whole kidney. TrxR2, Nrx, Grx1 and Prx5 were also detected in mTALs.

Proximal tubule cells can regenerate after an I/R insult (Nony and Schnellmann, 2003), (Molitoris and Marrs, 1999). The amounts of Grx2, Prx3 and Prx6 were significantly upregulated in proximal tubuli after the I/R insult.

Overexpression of these redoxins in HeLa and HEK293 cells, cultured for 24 h at 0.1 % O_2 and another 24 h at 20 % O_2, showed higher survival and proliferation rates and less oxidative damage to the DNA, detected by 8-OH-dG staining, compared to control cells (data not shown).

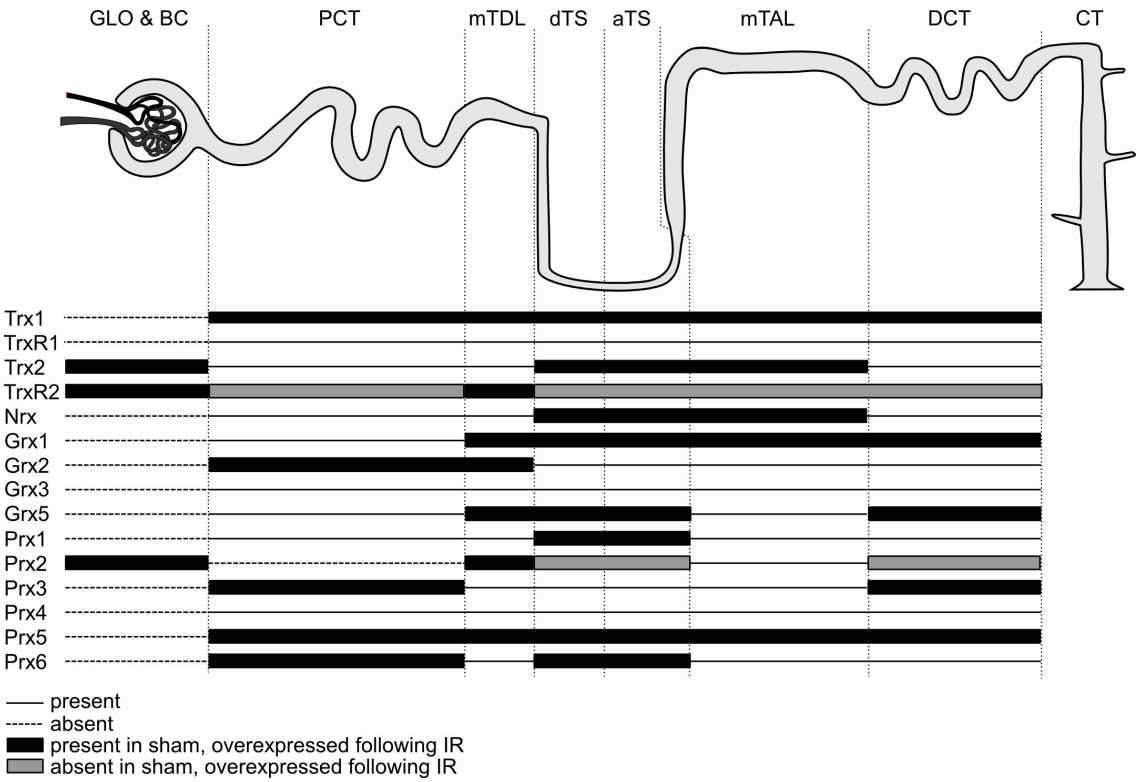

Figure 34: Segment specific distribution of Trx family proteins in sham and ischemic nephrons.

These data indicated that higher levels of these redoxins contribute to the regeneration process of these cells. As described above, many redoxins have been implicated in cell survival and proliferation, as well as the degradation of H_2O_2.

Mitochondrial Grx2 was shown to protect HeLa cells from cell death (Lillig et al. 2004), (Fernandes et al. 2009), inhibiting the release of cytochrome *c* (Enoksson et al. 2005). Thymoma cells, stably overexpressing mitochondrial Prx3 were characterised by resistance against hypoxia-induced production of H_2O_2 and apoptosis (Nonn et al. 2003). Using rat insulinoma cells with modified levels of Prx3, Wolf and coauthors demonstrated that the peroxidase reduced accumulated levels of H_2O_2 and prevented the induction of caspase-9 and caspase-3 (Wolf et al. 2010). Macrophages with depleted levels of Prx6 displayed increased levels of H_2O_2 and an elevated apoptosis rate (Wang et al. 2006). Overexpression of Prx6 in breast cancer cells enhanced the proliferation and invasion rate of the cells (Chang et al. 2007).

We included HEK293 cells in this study, because they are a generally accepted kidney-derived cell line, with neuronal origin. It is elusive to compare the cell line to the response of cells within the whole organ. Of course, this is a general problem of cell culture, because cells adapt to changes in the environment and might lose their original,

characteristic features. Furthermore, cells are usually cultured at 20 % O_2, an oxygen concentration, representing the concentration in the atmosphere and not necessarily physiological, intracellular concentrations. However, we have corroborated our hypothesis, demonstrating that redoxins can protect HEK293 cells from hypoxia-induced apoptosis. We have also analysed the responses of other cell lines in terms of cell viability and expression of Trx family proteins. Cell lines dramatically differed in the susceptibility towards oxygen deprivation. HeLa cells, HEK293 cells and also the rat pancreatic β-cell line INS1 were very resistant to the hypoxic conditions and could be cultured in an atmosphere containing 1 % or less oxygen for several days. The general expression pattern, as well as changes in expression of the Trx family proteins, following the hypoxic insult differed in these cell lines, confirming the complexity and the potential distribution to cell survival and the data of the animal models. The murine pancreatic β-cell line MIN6 was very susceptible to oxygen deprivation and could not be cultured at concentrations lower than 1 % oxygen. Exposing the cells to 1 % or 2 % oxygen for 12 h, led to a decrease in cell viability of around 40 %.

Cells have developed response mechanisms to cope with low oxygen concentrations. HIF-1 is a transcription factor, regulating the gene expression under hypoxic conditions. It consists of two subunits, α and β. Under normoxic conditions, HIF-1α is degraded by the proteasome, whereas HIF-1β is not. Upon hypoxia, HIF-1α is hydroxylated by oxygen-sensing prolyl hydroxylases (PHD), protecting it from ubiquitination. ROS and RNS can stabilise HIF-1α, by modifying PHDs posttranslationally, for instance by nitrosylation (Metzen et al. 2003), (Mansfield et al. 2005), (Sanjuán-Pla et al. 2005). Another regulatory mechanism of the transcription factor is the generally increased translation due to phosphorylation of essential proteins such as by phosphatidylinositol 3 kinase/Akt-dependent or MAPK signaling pathways (Semenza, 2003). Stress- and mitogen-activated protein kinase pathways generally constitute a cellular response mechanism to cope with alterations in the O_2 levels. Different studies revealed that the overexpression of Trx1 increased HIF-1α levels in cells grown under normoxic and hypoxic conditions. Inhibiting TrxR1 activity in different cell lines blocked the activation of HIF-1α (Moos et al. 2003). Zhou and coworkers described a diverse regulation of HIF-1α by cytosolic Trx1 and mitochondrial Trx2. Overexpression of Trx1 increased the levels of HIF-1α by activating Akt-dependent translation, whereas overexpression of Trx2 prevented or diminished hypoxia-induced HIF-1α accumulation (Zhou et al. 2007). In addition, Trx1

might be involved in HIF-1α-degradation during reoxygenation (Jewell et al. 2001). In our MIN6 model, the cytosolic Trx system decreased upon oxygen deprivation, whereas the mitochondrial Trx system increased. This study is, to our knowledge, the first study showing that both systems responded oppositional to a stimulus, even though different functions of the proteins have already been described.

Since we have not analysed the expression of HIF-1α, we can only speculate at this point. Due to the elevated expression of Trx2 it is possible that HIF-1α expression is attenuated, preventing an adapted stress response. This effect might be supported by the downregulation of TrxR1 and the secretion of Trx1. We have used medium of MIN6 cells, exposed to hypoxia, as well as recombinantly expressed Trx1 in an assay measuring the migration of macrophages through an affigel-matrix. In the presence of Trx1, the migration rate of macrophages, isolated from mice, was reduced, compared to control samples without added Trx1, implying a potential function in preventing the initiation of an inflammatory response. This effect could be explained by the inhibition of the macrophage migration inhibitor factor (MIF), a pro-inflammatory protein released from immune cells. Various studies have analysed Trx1 and MIF, revealing different potential mechanisms of regulation. Son and coworkers demonstrated a direct association between the two proteins (Son et al. 2009), inhibiting the release of MIF or directly regulating protein expression (Sato et al. 2006), (Nakamura, 2008).

The data on the Trx systems were confirmed in a mouse model for β-cell transplantation, where mice were treated with streptozotocin to induce type-1 diabetes and received a transplantation of β-cells of a healthy donor into the portal vein. Trx1 and TrxR1 were downregulated in the hypoxic state, i.e. after 30 min, whereas Trx2 and TrxR2 were upregulated. 120 days after the procedure no significant changes in protein expression were detected.

However, we were not able to quantify the levels of Trx1 in the plasma of diabetic animals prior or post β-cell transplantation using the Western blot technique. In order to analyse plasma levels of redoxins, it is desirable to establish more specific sandwich ELISAs.

We have furthermore analysed the expression and phosphorylation of various MAP kinases. The MAP kinase cascade generally includes three core signaling molecules. MAP kinase kinase kinases, such as Ask1, sense the degree of stress-induced damage in upstream signaling events and determine the cell fate by regulating MAP kinase kinases and MAP kinases, which then phosphorylate substrates as the effector proteins

(Matsuzawa and Ichijo, 2008).

Phosphorylation and redox signaling do not only constitute similar, rapid mechanisms regulating cellular functions, but they were furthermore shown to be connected at different points. As mentioned before, tyrosine phosphatases can be regulated via posttranslational modifications at a redox-active cysteine, which is essential for catalysis. And also MAP kinases can be redox regulated, i.e. being kept in an inactive state by a member of the Trx family of proteins. In a similar way as the regulation of Ask1 by Trx1, JNK is kept in an inactive state by GST-π1 in unstressed cells. In the presence of H_2O_2 for instance, GST dissociates and JNK is activated (Adler et al. 1999). Activation of p38, JNK and ERK was analysed in MIN6 cells following hypoxia and reoxygenation. The ERK pathway transduces extracellular stimuli into cascades of protein phosphorylation and activation, regulating cellular processes including proliferation and differentiation. ERK1 and ERK2 share similar protein sequences, but were shown to regulate different functions due to numerous substrates in distinct compartments (Lloyd, 2006). ROS act mitogenic, by inducing ERK signaling via receptor activation, i.e. phosphorylation (Burdon, 1995). Furthermore, ERK1/2 can function as anti-apoptotic factors, initiating cell survival following oxidative injury (Guyton et al. 1996).

JNK and p38 are also referred to as stress-activated protein kinases (SAPK), because they are activated by various types of stress. Even though, the activation of JNK and p38 and the inhibition of ERK was shown to be required for the induction of apoptosis, for instance in neuron like PC-12 cells (Xia et al. 1995), SAPK activation might also be important for cell proliferation and cell survival (Matsuzawa and Ichijo, 2001). There are two ubiquitously expressed isoforms of JNK, JNK1 and JNK2, and the brain-specific JNK3, which execute similar, but also different functions. JNK was shown to act in the same pathway as Ask1. Liu *et al.* for instance demonstrated that Tnf-α activates JNK1, but not JNK2, inducing apoptosis in mouse fibroblasts lacking either *Jnk1* or *Jnk2* (Liu et al. 2004).

Overexpression of Trx1 and Trx2 affected all analysed MAP kinases. Trx1 increased the levels of phosphorylated ERK1/2 under normoxic conditions, inducing proliferation – a known function of the oxidoreductase. In addition, Trx1 overexpressing cells displayed decreased levels of phosphorylated JNK1/2 upon hypoxia and phosphorylated levels of p38 under hypoxia and reoxygenation. Inhibition of SAPK-signaling inhibited the induction of apoptosis. Cells overexpressing Trx2, showed a similar phosphorylation

pattern of the MAP kinases. However, the extent of the phosphorylation of the different MAP kinases by Trx1 and Trx2 differed, which could be explained by different protein accessibility in different compartments. No direct interaction between the oxido-reductases and the MAP kinases was analysed. So far, no direct redox regulation of kinases was shown. It is possible that the effects seen in the downstream proteins of the signaling pathways, are due to a regulatory mechanism of Ask1 by Trxs (Saitoh et al. 1998).

MIN6 cells showed unique alterations in protein levels of the Trx family proteins upon hypoxia and reoxygenation. Cytosolic and mitochondrial proteins of the Trx system and the Prxs, responded not only differently, but often even oppositional. This organelle-specific response to the hypoxic insult underlines the concept of compartmentalised redox signaling and the importance of the different proteins and isoforms of the Trx family. However, the protein levels of all four Grxs were reduced during hypoxia and reoxygenation. Grxs are known as GSH-dependent oxidoreductases. Total GSH levels were significantly decreased upon hypoxia in MIN6 cells, but recovered during the reoxygenation period. The same pattern was detected for the two dithiol Grxs, Grx1 and Grx2. On the contrary, the protein levels of the two monothiol Grxs, Grx3 and Grx5, did not seem to recover during reoxygenation. Monothiol redoxins have not been shown to be catalytically active and therefore do not rely on GSH in the same way as dithiol proteins. However, GSH is needed for cluster coordination (Haunhorst et al. 2010). The total Grx2 levels, also including cytosolic Grx2c, were significantly downregulated. At the same time the cytosolic Prx2 and Prx6 were downregulated, constituting another indication for potential protein interaction.

The reduction in the levels of the peroxidases might lead to the induction of apoptosis following the hypoxic insult. Tulsawani and coauthors exposed retinal ganglion cells to hypoxia and detected a reduced expression of Prx6, with increased levels of ROS, activation of NFκB and the induction of apoptosis. Moreover, they demonstrated that the overexpression of Prx6 in these cells reduced hypoxia-induced cell death. These data also coincide with our findings on Prx6 in the renal I/R model. The peroxidase was upregulated in tubule cells, which can regenerate following an I/R insult and the overexpression of Prx6 in HEK293 and HeLa cells increased proliferation and protected cells against hypoxia-induced apoptosis. Prx3 and Grx2 were also upregulated in the tubule cells and acted proliferative, as well as anti-apoptotic in the cell models. Interestingly, these two proteins are also downregulated in MIN6 cells following

hypoxia and reoxygenation. The cytosolic Prx1 responded differently. It was upregulated during hypoxia, pointing to a different function of the peroxidase. Kim and coworkers discovered the upregulation of Prx1 in a different cell model, A549 cells, following 4 h hypoxia and 2 – 24 h reoxygenation (Kim et al. 2007). Different studies demonstrated that Prx1 was overexpressed in several cancer cell lines (Yanagawa et al. 1999), (Yanagawa et al. 2000). Many cancer cells can grow under hypoxic conditions with the degree of hypoxia correlating to tumor growth and resistance to radiation therapy (Harris, 2002). Chen and coworkers have shown that these tumor features correspond to the expression of Prx1 (Chen et al. 2006). Kim and coworkers did not only confirm this proliferative and anti-apoptotic function of Prx1, but furthermore explained it by the finding that Prx1 inhibited the JNK-signaling pathway. This inhibition was not dependent on the peroxidase activity, but rather on the interaction with the GST-JNK complex (Kim et al. 2006), (Kim et al. 2007).

Prx5 increased during hypoxia, but decreased following reoxygenation and thereby showed a similar response as Prx1. This either points to specific functions during hypoxic conditions or to different contributions in the response to hypoxia and reoxygenation. This has already been described above for the regulation of HIF-1α by Trx1. During hypoxia it induced the expression of HIF-1α, whereas during hypoxia it functioned in protein degradation (Zhou et al. 2007), (Jewell et al. 2001).

Prx2 levels were decreased upon hypoxia, whereas Prx4 was upregulated, implying specific functions for the peroxidases in MIN6 cells. So far, no studies on the function of those proteins during hypoxia have been described. However, Prx2 was implicated in the regulation of ERK, JNK and p38 in response to Tnf-α-induced H_2O_2 production (Kang et al. 2004). Both peroxidases were shown to be expressed in different cell types of the pancreas. Prx4 was expressed in the insulin producing β-cells, whereas Prx2 was located outside the insulin producing islets, in the exocrine pancreas. Figure 35 is part of the Mouse Atlas (Godoy et al. 2011a), showing the distinct expression and distribution of numerous Trx family proteins in the mouse pancreas.

In all analysed cell and animal models, the Trx family proteins showed a distinct expression and distribution profile and furthermore responded species-, tissue- and cell type-specific to oxidising and hypoxic conditions, implying distinct functions and contributions to regulating or determining the fate of a specific cell.

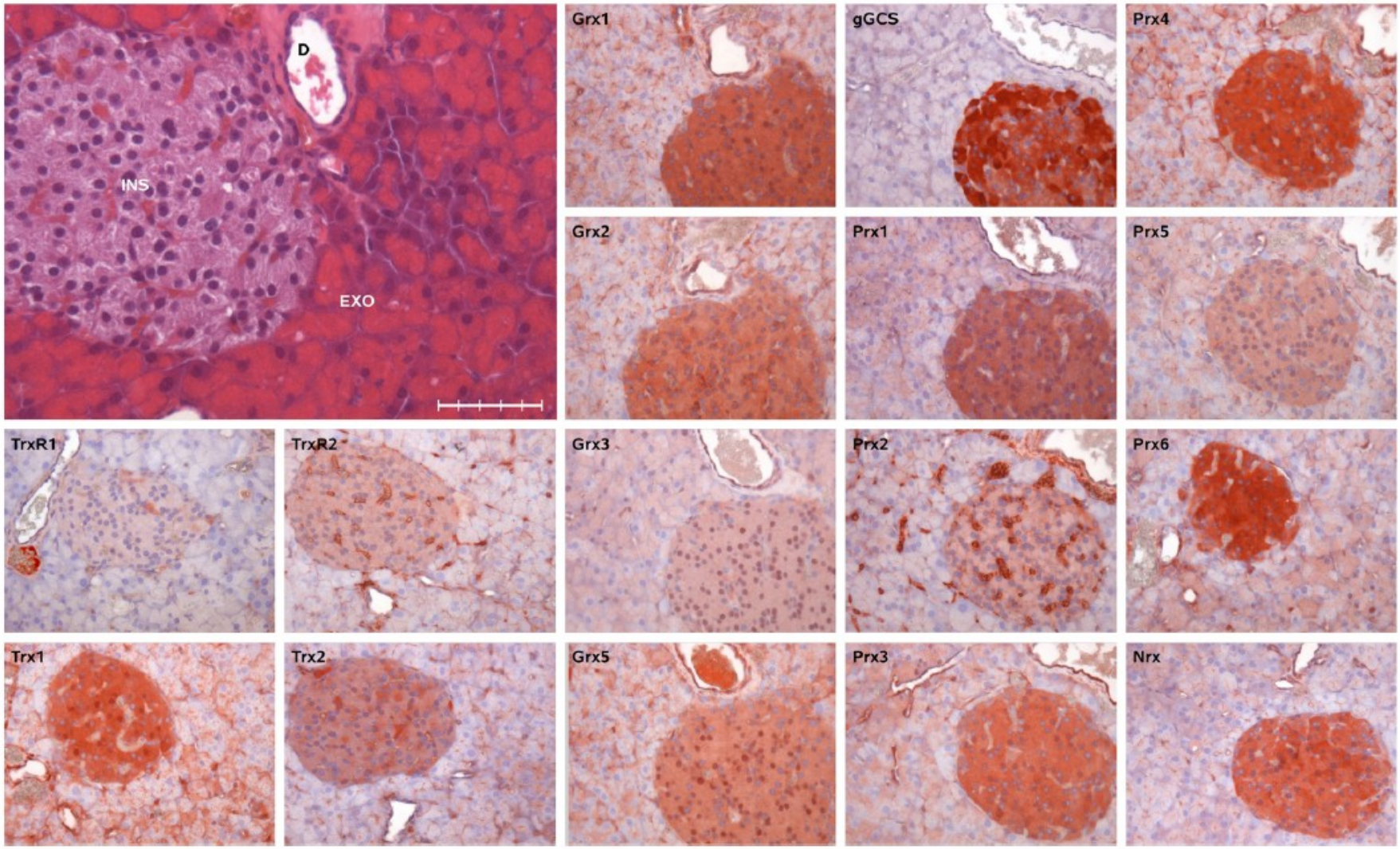

Figure 35: Trx family proteins in the mouse pancreas. D=duct, EXO=exocrine pancreas, INS=islet of Langerhans, Objective: 25x, scale bar: 50 µm (image from the "Mouse Redox Atlas", Godoy et al. 2011a)

4.3 Main conclusions from the papers

Part of the results presented in this thesis have already been published or have contributed to the preparation of manuscripts for later submission and publication. The main conclusions from the individual papers are as follows:

Paper I: **Hanschmann E.M.**, Lönn M.E., Schütte L.D., Funke M., Godoy J.R., Eitner S., Hudemann C. and Lillig C.H. Both thioredoxin 2 and glutaredoxin 2 contribute to the reduction of the mitochondrial 2-Cys Peroxiredoxin Prx3. J. Biol. Chem. 285(52):40699-705 (2010)

- Grx2 donated electrons to the 2-Cys Prx3 in a dithiol mechanism *in vitro*, but not to the atypical 2-Cys Prx5.
- Silencing the expression of either Grx2 or Trx2 in HeLa cells by RNA interference did not lead to the accumulation of oxidised Prx3. Only simultaneous knock-down led to an increase in oxidised Prx3, pointing to an *in vivo* function as electron donor for both oxidoreductases.
- The expression of Prx3 in mouse tissues was either linked to the presence of TrxR2/Trx2, GSH/Grx2 or TrxR2/Grx2, suggesting a distinct and tissue-specific contribution of the individual systems to the redox state of the mitochondrial peroxidase.

Paper II (manuscript): Haunhorst P.*, **Hanschmann E.M.***, Bräutigam L., Stehling O., Lill. R., Mühlenhoff U., Berndt C. and Lillig C.H. Glutaredoxin 3 (PICOT) functions in iron homeostasis and is essential for hemoglobin maturation in zebrafish (*shared first authorship)

- Grx3-depleted 48 hpf zebrafish embryos showed an impaired maturation of hemoglobin.
- Grx3-depleted 48 hpf zebrafish embryos were characterised by reduced activities of the iron-sulfur protein aconitase and the heme protein cytochrome *c* oxidase.
- Knock-down of Grx3 in HeLa cells led to an iron deprivation phenotype, even though iron supply and uptake were not affected. The TfR was upregulated,

ferritin and the iron regulatory protein IRP1 were downregulated. IRP2 expression was not affected.

- Enzymatic activities of cytosolic FeS proteins, as well as iron-dependent proteins in mitochondria, were significantly downregulated.

Paper III: Aon-Bertolino M.L., Romero J.I., Galeano P., Holubiec M., Badorrey M.S., Saraceno G.E., **Hanschmann E.M.**, Lillig C.H. and Capani F. Thioredoxin and Glutaredoxin system proteins-immunolocalization in the rat central nervous system. Biochim Biophys Acta. 1810(1):93-110 (2011)

- All proteins, Trx1, Trx2, TrxR1, TrxR2, Txnip, Prx1, Prx2, Prx3, Prx4, Prx5, Prx6, Grx1, Grx2, Grx3, Grx5 and γGCS were detected throughout the rat CNS and showed a tissue- and cell type-specific expression pattern, indicating distinct protein functions and complex crosstalk between the family members.

Paper IV: Godoy, J.R., Oesteritz, S.*, **Hanschmann, E.M.***, Ockenga, W., Ackermann, W. and Lillig, C.H. Segment-specific overexpression of redoxins after renal ischemia and reperfusion: protective roles of glutaredoxin 2, peroxiredoxin 3, and peroxiredoxin 6. Free Radic. Biol. Med. 51(2):552-61 (2011), (*contributed equally to this work)

- I/R kidneys showed significantly increased levels of Trx1, Trx2 and Grx5, whereas Grx1 was decreased. Contralateral kidneys were characterised by increased Grx5 and Prx6 levels, while protein amounts of TrxR2, Prx4 and Prx5 were decreased. These differences in both, operated and contralateral kidneys provided evidence not only for local but also for systemic implications following the I/R insult.
- Immunohistochemical analysis of Trx family proteins (Trx1, Trx2, TrxR1, TrxR2, Nrx, Prx1, Prx2, Prx3, Prx4, Prx5, Prx6, Grx1, Grx2, Grx3 and Grx5) showed segment-specific alterations induced by the ischemic insult.
- Grx2, Prx3 and Prx6 were upregulated in proximal tubule cells, a cell type, which can regenerate after an I/R insult. Overexpression of Grx2, Prx3 and Prx6 in HeLa cells led to lower oxidative damage to the DNA, compared to controls.

- Overexpression of Grx2, Prx3 and Prx6 in HEK293 cells revealed a higher cell survival and proliferation rate, compared to controls.
- Following I/R, Grx1 accumulated at the apical side of distal convoluted cells and was specifically secreted into the urine.
- The differences in both the basal equipment and the segment-specific responses of the redoxins may contribute to the distinct susceptibilities and regeneration processes of the various segments of the nephron to the I/R insult.

Paper V (Manuscript II): Hanschmann E.M., Rawat N., Linn T. and Lillig C.H. The cytosolic and mitochondrial Trx systems act diverse in a model for β-cell transplantation (in preparation)

- The cytosolic and the mitochondrial Trx systems responded differently to hypoxic conditions in the pancreatic β-cell line MIN6. Protein levels of cytosolic Trx1 and TrxR1 strongly decreased, whereas the levels of the mitochondrial Trx2 and TrxR2 increased.
- Immunohistological analysis of mice with streptozotocin-induced diabetes with subsequent transplantation of pancreatic β-cells showed a lower signal for Trx1/TrxR1 and a stronger staining for Trx2/TrxR2 in the hypoxic state, 30 min after transplantation, than control animals. After 120 days of reoxygenation or rather regeneration, the expression pattern of the transplanted cells was similar to healthy pancreatic β-cells.
- Overexpression of Trx1 and Trx2 increased the levels of phosphorylated ERK, initiating cell proliferation and differentiation.
- Overexpression of Trx1 and Trx2 decreased the levels of phosphorylated SAPK/JNK and p38, preventing cell death.
- Trx1 was specifically secreted from MIN6 cells upon the hypoxic insult.
- Trx1 inhibited the migration of macrophages, potentially preventing the initiation of an inflammatory response *in vivo*.

4.4 Significance and future perspective

Nowadays, the redox state of a protein is generally accepted as an essential mediator of protein function and the term redox signaling is widely applied in the field. Concentrations and localisation of ROS and Trx family proteins can control if a cell undergoes proliferation or apoptosis. However, we are only beginning to understand the general mechanisms and the overall significance of redox signaling.

This thesis has substantially contributed to broaden and extend the knowledge on the thioredoxin family proteins – in physiology and disease.

We have identified human Grx2 as electron donor for the mitochondrial Prx3, providing novel insights into crucial redox signaling events and revealing yet another crosslink between members of the Trx family. It remains to be analysed if the cytosolic/ nuclear isoforms Grx2b and Grx2c (Lönn et al. 2008) can donate electrons to the cytosolic peroxidases. Since these isoforms are cancer/testis specific, it would be interesting to determine if these redoxins contribute e.g. to the increased proliferation of cancer cells and resistance to chemotherapy.

We have discovered a novel function in intracellular iron homeostasis and heme maturation for Grx3 in zebrafish and human. We suggest that Grx3 functions in iron trafficking through the cytosol. The general mechanism and potential target proteins need to be identified and analysed. Grx3 was shown to exist as dimer coordinating two [2Fe2S] clusters (Haunhorst et al. 2010). It would be interesting to analyse whether the function in iron regulation depends on these cofactors. Trying to rescue the Grx3 depletion phenotype in HeLa cells by overexpressing the wildtype protein, as well as deletion-mutants lacking the monothiol domain(s) and thus the ability to constitute FeS clusters, might answer this question. In addition, one could use co-immunoprecipitation to detect new interaction partners in general, but also to analyse if there is a direct interaction between Grx3 and IRP1, similar to the interaction described for Grx4 and the transcriptional activator Aft1 in *S. cerevisiae* (Rutherford et al. 2005). Grx4 also coordinates an iron cofactor, which is essential for the regulation of Aft1, suggesting a potential function as iron sensor (Pujol-Carrion et al. 2006). The fluorescence resonance energy transfer technique could be applied to analyse and define the spatial relationship between Grx3 and IRP1.

The collaborative projects of human (Dammeyer and Arnér, 2011), mouse (Godoy et al. 2011a) and rat protein (Aon-Bertolino et al. 2011) atlases were a successful approach to

analyse Trx family proteins in numerous organs, tissues and cell types. We retrieved new data on single proteins, networks between redoxins and potential new functions. Mainly these studies in fact revealed that protein expression and cellular distribution were tissue-, cell type- and species-specific. Overall, we believe that these studies represent a basis and a point of reference for future disease-orientated studies.

We confirmed the above described complexity of the redox regulatory networks of the Trx family proteins in models for perinatal asphyxia, renal I/R injury and the transplantation of pancreatic β-cells - conditions induced by the lack or depletion of oxygen.

So far, Trx family proteins have not been analysed in perinatal asphyxia, which constitutes a major cause of death worldwide (Lawn et al. 2005). Even though the analysis of the hippocampus, one of the most affected regions of the brain by the hypoxic insult, showed the importance of the redoxins in general, it is desirable to analyse more tissues and cell types for protein expression and distribution. It would be interesting to include transgenic animals with depleted or increased expression of redoxins in further experiments, analysing them for susceptibility to hypoxia, cell survival, regeneration and brain function, compared to wildtype animals.

Renal I/R injury constitutes the leading cause of acute renal failure and has been correlated to the progression and development of chronic kidney diseases (Weight et al. 1996), (Sutton et al. 2002). We believe that the differences in the physiological levels and the segment-specific responses to the I/R insult contribute to the different susceptibilities and the regeneration of distinct cell types within nephron segments. Grx2, Prx3 and Prx6 were shown to increase the cell number and decrease oxidative damage to the DNA in HeLa and HEK293 cells. However, there is no cell model which can mimic the conditions within a whole organ. Therefore it is essential to analyse the protective or even regenerative effects of the redoxins in an animal model. Again, it would be interesting to run studies on transgenic animals. For the first time, we have shown that Grx1 was specifically secreted into the urine following the I/R insult. Further research, analysing Grx1 secretion in animal models and human patients might establish Grx1 as a potential biomarker for renal I/R injury.

A similar secretion was detected in murine MIN6 cells for Trx1 following a hypoxic insult, which needs to be confirmed in diabetic animals, analysing serum samples, shortly after transplantation. At the same time the potential extracellular function in preventing the immune response should be confirmed and the mechanism analysed.

The diverse response of the cytosolic and the mitochondrial Trx system upon hypoxia/ reoxygenation has never been described in any model, upon any stimuli, before. It is essential to investigate the underlying mechanism, as well as the potential functions. We have seen that the overexpression of Trx1 and Trx2 initiated the anti-apoptotic signaling pathways and inhibited pro-apoptotic pathways. However, simultaneous downregulation of Trx1 and upregulation of Trx2 should be included in further studies as well as the diverse condition.

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6. Appendix

Curriculum vitae

Personal information

name:	Eva-Maria Hanschmann
date of birth:	22/05/1983
place of birth:	Essen
nationality:	german

Education

1989-1993	Graf-Spee Schule (primary school), Essen
1993-2002	BMV Schule (gymnasium), Essen (degree: Abitur)
11/2002-04/2007	Studies of biology (degree: diploma/ int. M. Sc.) at the Ruhr-Universität Bochum, *Diploma thesis: "The role of human glutaredoxin 2",* The Medical Nobel Institute for Biochemistry, Department of Medical Biochemistry and Biophysics. Karolinska Institutet, Stockholm, Sweden, supervision: Dr. C. Berndt, Prof. MD, PhD A. Holmgren
05/2007-07/2008	Research assistant at the Department of Medical Biochemistry and Biophysics and the Department of Medicine, Karolinska Institutet and Karolinska Sjukhuset, Stockholm, Sweden, supervision Prof. MD, PhD A. Holmgren, PhD P. Gil
since 07/2008	PhD student at the Department of Clinical Cytobiology and Cytopathology, Philipps-Universität Marburg: *"Thioredoxin family proteins in physiology and disease"*, supervision: PD Dr. C.H. Lillig
	Practical course at the Instituto de Investigaciones Cardiólogicas (ININCA), Facultad de Medicina, Universidad de Buenos Aires, Argentina, "*Rat model for perinatal asphyxia*".
	Practical course at the Medical Nobel Institute for Biochemistry, Department of Medical Biochemistry and Biophysics. Karolinska Institutet, Stockholm, *"Oxidative stress – disease, methods, concepts"*

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Berndt C., Hudemann C., **Hanschmann E.M.**, Axelsson R., Holmgren A. and Lillig C.H. *How does iron-sulfur cluster coordination regulate the activity of human glutaredoxin 2?* Antiox. Redox Signal. 9(1):151-157 (2007)

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My scientific teachers

Berndt, Carsten (Dr.)	Karolinska Institutet, Stockholm
Faissner, Andreas (Prof. Dr.)	Ruhr-Universität Bochum, Germany
Gatermann, Sören (Prof. Dr.)	Ruhr-Universität Bochum, Germany
Gerwert, Klaus (Prof. Dr.)	Ruhr-Universität Bochum, Germany
Gil, Pedro (PhD)	Karolinka Sjukhuset, Stockholm
Hatt, Hanns (Prof. Dr. Dr.)	Ruhr-Universität Bochum, Germany
Hoffmann, Klaus-Peter (Prof. Dr.)	Ruhr-Universität Bochum, Germany
Hofmann, Eckert (Prof. Dr.)	Ruhr-Universität Bochum, Germany
Holmgren, Arne (Prof. Dr. Dr.)	Karolinska Institutet, Stockholm
Kirchner, Wolfgang (Prof. Dr.)	Ruhr-Universität Bochum, Germany
Kruse, Wolfgang (PD Dr.)	Ruhr-Universität Bochum, Germany
Kück, Ulrich (Prof. Dr.)	Ruhr-Universität Bochum, Germany
Lillig, Christopher Horst (PD, Dr.)	Philipps-Universität Marburg, Germany
Lübbert, Hermann (Prof. Dr.)	Ruhr-Universität Bochum, Germany
Narberhaus, Franz (Prof. Dr.)	Ruhr-Universität Bochum, Germany
Rögner, Matthias (Prof. Dr.)	Ruhr-Universität Bochum, Germany
Schlitter, Jürgen (PD Dr.)	Ruhr-Universität Bochum, Germany
Störtkuhl, Klemens (Prof. Dr.)	Ruhr-Universität Bochum, Germany
Stützel, Thomas (Prof. Dr.)	Ruhr-Universität Bochum, Germany
Weiler, Elmar (Prof. Dr. Dr.)	Ruhr-Universität Bochum, Germany

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